UNIVERSITY OF ALBERTA

PECTIN-WHEY PROTEIN INTERACTIONS IN WHEY-BASED TOMATO BEVERAGES

BY

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ABSTRACT

Three model systems using solutions of whey protein isolate (WPI) in water; mixed with pectin; or tomato juice, were produced at pH 3.3, 3.7, or 4.2 to study interactions of whey proteins and pectin.

This investigation confirmed the complexation of whey proteins and pectin at pH lower than 3.9 leading to the development of visual sedimentation. Whey proteins precipitated following heating below pH 3.9 but resisted coagulation and sedimentation at pH 4.2 in the presence of pectin. The pectin reactivity in tomato juice and its complexation with whey proteins at the pH range studied was illustrated indirectly by measuring sediment formation, its composition and the viscosity of WPI-tomato juice systems.

Incorporation of whey proteins had a favorable effect in the spray drying of tomato juice and in blends containing fresh whey at pH 3.3 and 4.2; however, visual sedimentation was observed in reconstituted mixtures due to the drying process.

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1. INTRODUCTION

1.1 Prospects for whey utilization

The by-product of cheese processing, fresh whey, is the product of the precipitation and separation of casein and fat from milk. Cheese production is a low-yield process since the amount of cheese obtained, only 10 to 20%, contrasts with the 80 to 90% of whey produced, creating the necessity for the processing or disposal of a huge amount of material. The degradation of the whey is a process involving a high biological oxygen demand due to its lactose and protein content. Consequently, its throwing into rivers or other water sources results not only in a loss of high value nutrients but also in the production of a high degree of pollution (Sienkiewicz and Riedel, 1990; Smithers et al., 1996).

Whey production, close to 120 million tones / year around the world, results in the creation of almost 0.7 million tones of high quality protein as well as other nutrients such as lactose, minerals, and vitamins annually (Bylun, 1995b; Smithers et al., 1996). It was estimated that approximately 50% to 60% of the total cheese whey produced is being used in yoghurt, ice-creams, soft drinks, bread, infant foods and animal feed, while 40% to 50% of the remaining whey is thrown away since it is considered to be a secondary by-product without great nutritional value in spite of the protein deficiency in many countries (Mott et al., 1999; Bylun, 1995b).

Because whey is mainly water, its utilization directly as a beverage could be the most logical option. However, employing fresh whey as a component of any food involves the necessity to treat it thermally, which causes undesirable effects in the final product due to

the high susceptibility of whey proteins to coagulate and form sediments after heating (Jelen and Buchheim, 1984; Mangino et al., 1987). Nevertheless, despite the effect of high temperature on whey proteins, Jelen and Bucheim (1984) overcame the sedimentation problem of whey protein solutions by heating them at a pH below 3.9.

Quite a different effect was noted by Jelen and Currie (1987), when analyzing different types of commercially produced whey beverages containing fruit juices and added sugar when a heat treatment was applied. A clear sedimentation behavior was seen even at pH range of 3.0-3.9 (Jelen and Currie, 1987). An important additional factor in the heat stability of whey proteins is related to the presence of calcium (Patocka et al., 1986). Hence, acid whey, obtained from the use of organic or mineral acids, with its higher calcium content than sweet whey, produces less sedimentation when used in the preparation of fresh whey drinks.

On the other hand, since the utilization of fresh whey entails another associated problem, its unpleasant flavour, and considering that the optional utilization of heavily flavoured whey has limited application in healthy products, the simplest alternative to hide the flavour of the whey is to mix it with different types of fruit juices (Carunchia et al., 2003). As an example, the commercial fresh whey-based drinks traditionally produced in several European countries (in particular in Germany, Austria, Holland and Switzerland), correspond to two types: clear drinks, basically protein free beverages made from ultrafiltered permeate or deproteinated whey; and cloudy drinks, containing untreated whey, fruit juices or flavoured ingredients (Jelen and Currie, 1987).

An additional detrimental factor in the utilization of liquid whey as a basic component of fresh whey-based products is the handling of

great volumes of whey to be processed and stored. Modern membrane separation technologies in use for already several years, led to the development of whey protein concentrates (WPC) and whey protein isolates (WPI) in powdered form, having an approximate protein content of 70% and 90% on a dry matter basis respectively and a high stability in storage, thanks to their low moisture content, which ranges from 4 to 5%. WPCs and WPIs are produced by spray drying the retentates after the ultrafiltration of whey (Pasin and Miller, 2000; Harper, 2000).

In this regard, the commercial production and use of the WPCs and WPIs represent an innovative option for the large scale utilization of whey proteins in human foods. Milk, yoghurt, cheese, instant mashed potatoes, ice-cream, whipping cream, dairy desserts, bakery products, and certain types of pasta are among the products usually fortified by the addition of WPCs and WPIs (Sienkiewicz and Riedel, 1990; Kosaric and Asher, 1982). WPCs and WPIs have been used also as protein supplements in children's diets for many years through their direct addition into canned stews, whey-based beverages, and dry puddings in government food programs in some South American countries (Salinas, personal experience).

A new worldwide consciousness concerning the consumption of healthy foods, has led to the recent development and production of functional foods. While the whey proteins have an unquestionable place as ingredients in functional foods due to their nutritional quality, tomato, a long utilized fruit in human consumption, is being highly appreciated nowadays as an excellent source of lycopene. Italian researchers from the University of Milan have reported that a daily regular consumption of Lyc-o-Mato, a commercial tomato drink providing lycopene among other carotenoids, has a positive effect against the atherosclerosis and cardiovascular disease (Riso et al., 2006). Besides lycopene and other

carotenoids, tomatoes are an important source of other nutrients such as Vitamin A, Vitamin C, iron, and potassium (Gould, 1983; Barret et al., 1998). Polysaccharides in tomato juice comprise close to 0.7% with pectins and arabinogalactans composing about 50% of this amount (Miladi et al., 1969), and although pectins do not have nutritional value in human diet they play a significant role in the viscosity of tomato juice, tomato paste, and other processed tomato products.

While tomato and fresh whey-based drinks of several types have been produced in Europe and North America for many years, little research has been reported on tomato fortified products with whey protein concentrate. Because investigations on the interactions of whey proteins and pectin are still scarce, this research project was designed to investigate in a more detailed way the interactions between whey proteins and pectin, to generate new knowledge leading to possible development of tomato juice drinks containing whey or whey proteins.

This study was based on the previous work of Devkota (1991) who developed a successful prototype tomato and fresh whey-based drink by mixing 50% fresh whey and 50% commercial tomato juice, which showed no significant difference when tested for the acceptance comparing with the commercial tomato-based drink clamato.

1.2 Research objectives

The overall purpose of this project was the investigation leading to development of a tomato juice drink containing cheese whey or whey proteins in the form of:

a) mixture of fluid whey and tomato juice

b) tomato juice fortified with whey protein isolate

The specific objectives are described as follows:

1) Study interactions between high methoxy (HM) industrial pectin and whey protein isolate (WPI) at pH 3.3, 3.7, or 4.2, in water model and real systems, and in unheated and heated conditions to reproduce the thermal processing required for industrial food safety.

2) Study interactions between tomato pectin and whey proteins in mixtures of tomato juice and WPI at pH 3.3, 3.7, or 4.2.

3) Evaluate the spray drying of a prototype whey protein concentrate-tomato juice drink and some physico-chemical properties of the resulting powder.

2. LITERATURE REVIEW

2.1 Whey

Whey, a liquid by-product of cheese making, used to be disposed of as forage or wasted as a useless product in former times, despite its superior quality proteins, lactose, vitamins and minerals content (Rektor and Vatai, 2003). The fresh whey produced from the processing of cheese using rennet casein is known as sweet whey, having a pH higher than 5.8, while whey from precipitation of casein by organic or mineral acids is known as acid whey, with a pH usually lower than 5.0 (Gallardo-Escamilla et al., 2005).

The general composition of sweet and acid whey varies. While for rennet whey, the protein content is slightly higher than for acid whey, a further considerable difference can be noted with regard to the calcium content, which in acid whey is higher than in rennet whey and partially occurs as lactate in solution (Bylun, 1995b; Gallardo-Escamilla et al., 2005; Sienkiewicz and Riedel, 1990).

Whey proteins are quickly absorbed since they comprise a high concentration of essential amino acids with Glutamic acid, Lysine, Aspartic acid, and Leucine being the most important. This fact, in conjunction with the importance of some types of whey as source of minerals such as calcium, makes it a highly nutritious supplement (Pasin and Miller, 2000; Glass and Hedrick, 1976). The general compositional estimates for acid and sweet whey are shown in Table 2.1.

Table 2.1 Approximate composition of whey (generalized)*

Component	Sweet whey (% by weight)	Acid whey (% by weight)
Total solids	6.4	6.5
Water	93.6	93.5
Fat	0.05	0.04
Protein	0.91	0.76
Lactose	4.8	4.9
Ash (Minerals)	0.50	0.80
Calcium	0.043	0.12
Phosphorus	0.040	0.065
Sodium	0.050	0.050
Lactic acid	0.05	0.40

*Adapted from Bylun (1995b) and Gallardo-Escamilla et al. (2005)

2.1.1 Whey processing

Fresh whey has been used in cattle feeding for many years even if it has caused storage and transport problems. The evaporation or drying of fresh whey was implemented as the first processing alternative, and allowed the use of whey powder as a direct food source or as a component of animal feed mixtures. In more recent years, the membrane

technology was developed and is widely used in the processing of milk, whey and other dairy products (Sienkiewicz and Riedel, 1990; Bylun, 1995b). There are several main processes applied to whey or its components.

Concentration and drying. A first preconcentration stage is usually made in a reverse osmosis (RO) tubular plant and subsequently a second concentration stage is carried out in a single or double-effect falling film evaporator. Whey is concentrated close to 50% total solids content, and then cooled to 15°C under continuous stirring for some hours to crystallize lactose in the form of the smallest sized crystals (Bylun, 1995b).

Spray drying is the technique extensively used today for the drying of liquid sweet whey. Small crystals of lactose with non-hygroscopic characteristics are a crucial condition to avoid the formation of lumps and thus achieve the successful spray drying of whey (Bylun, 1995b). Acid whey is a hard material to dry using this method due to its high content of lactic acid which causes agglomerations and lumps, obstructing the nozzle or atomizing wheel. The neutralization with NaOH or $Ca(OH)_2$ to pH 6, the co-drying with carrier substances or the gas injection are alternative required treatments for the spray drying of acid whey (Sienkiewicz and Riedel, 1990; Bylun, 1995b) .

Recovery of components. The usual methods for the recovery of proteins and other components from whey are membrane separation, chromatographic processes, and precipitation. The recovery of proteins from liquid whey is made by ultrafiltration (UF) and the dried retentates of ultrafiltered whey are called whey protein concentrates (WPC). WPCs produced by UF usually have different protein levels varying from 50% up to 85% on a dry matter basis. Diafiltration is a supplementary process consisting of the "washing" of low molecular components with water to

increase the removal mainly of lactose and minerals, resulting in the production of whey protein isolates (WPI) with protein content higher than 90% (Harper, 2000; Pasin and Miller, 2000; Bylun, 1995b).

Defatting of the ultrafiltered retentate of WPC is sometimes made in a microfiltration (MF) plant. The fat content of the WPC is reduced from 7.2% to 0.4%, fat globule membranes and bacteria are also concentrated in the retentate while the defatted permeate is treated in a secondary UF station where concentration is increased and diafiltration is then applied (Bylun, 1995b).

Chromatographic isolation is the method used to recover Lactoferrin (LF) and Lactoperoxidase (LP) from whey on an industrial scale. The process is based on adjustment to the isoelectric point of both proteins which is in the pH 9.0-9.5 range. At the standard pH 6.6 of sweet whey LF and LP have a positive charge although the remaining proteins are negatively charged. Then LF and LP are easily isolated using cation exchange resin. The resulting product contains 1% protein which, after UF and diafiltration, produces a protein with purity close to 95% (Bylun, 1995b).

Electrodialysis treatment is used to decrease the mineral content of fresh whey. The process consists of the flow of whey through electrically charged selective permeable membranes. Membranes containing negatively charged groups are called cationic membranes and those with positively charged groups are called anionic membranes. These semi-permeable structures allow the passage of cations or anions, Na^+, K^+, and Cl^-, depending on the selective process to be applied. The optimal electrodialysis of whey is obtained at pH 4.65, since at this pH the net charge of proteins is close to zero (Sienkiewicz and Riedel, 1990). An alternative efficient method, electro-osmosis, a modification of reverse osmosis employing an external electric field, also has been applied for

demineralization of whey in USA, Japan and Norway from almost 40 years (Powles and Murad, 1998; Sienkiewicz and Riedel, 1990).

Ion exchange produces up to 99% demineralized whey after consecutive flow through cation and anion exchangers. This demineralized whey can be immediately dried, producing a non-hygroscopic powder (Sienkiewicz and Riedel, 1990). Lactose is usually recovered from whey by crystallization and the subsequent separation of crystals by centrifugation and drying. Following fluidized-bed drying, the remaining moisture content of lactose is 0.1-0.5% (Bylun, 1995b).

2.1.2 Uses of whey

Whey powder from dried rennet whey, and dried acid whey after adjustment of the pH to 6.0, is often used to replace skim milk powder in a proportion of 10-25% in confectionary products, according to Sienkiewicz and Riedel (1990). Some advantages in the use of whey powder in these kinds of products are an enhancement of colour, the development of soft coagulum in a heated condition by using whey proteins and caseinate mixtures, better water retention, and a higher mineral content. Nowadays, the developments in fractionation and recovery technologies made possible the commercial production of WPCs and WPIs which have wide applications as food ingredients (Sienkiewicz and Riedel, 1990).

WPCs and WPIs have been usually used for protein fortification of milk and other products such as yoghurt, cheese, dry potato puree, baby foods, sausages, dry soups, salad dressing, ice-cream, bakery products and pasta. Mixtures of WPC and carragenans, pectins, agar or starch may be used in the production of desserts, puddings and jellies. In the case of yoghurt, the addition of WPC stabilizes the coagulum having an

anti-syneresis effect in addition to the increasing of the protein content (Bylun, 1995b; Sienkiewicz and Riedel, 1990).

Whey is used in the production of the Italian-style whey cheese ("Ricotta Cheese") by thermal denaturation. A different option is the addition of denatured or separated whey proteins to cheese milk to increase the yield of the cheese process (Knopp, 1988). Norwegian cheeses of the "Mysost" type are produced from concentrated whey (whole whey) with optional inclusion of milk fat. One of the main components of this product is lactose (Jelen, 1992; Sienkiewicz and Riedel, 1990). On the other hand, "Ziger" whey cheese (from former Yugoslavia) is obtained from the precipitation of casein and whey proteins by heating and acid addition. It has over 51% whey protein (weight basis) and 15% fat in dry matter (McSweeney et al., 2004; Sienkiewicz and Riedel, 1990). Whey cheeses may be classified into three different groups according to the fat content: cream whey cheese (> 33% fat in dry matter), whey cheese (10%-33% fat in dry matter) and skim whey cheese (< 10% fat in dry matter) as suggested by Sienkiewicz and Riedel (1990).

2.2 Whey proteins

2.2.1 Main types of whey proteins in milk

The main whey proteins are β-lactoglobulin, α-lactalbumin, Bovine Serum Albumin (BSA), immunoglobulins, and proteose-peptones according to De Wit (1998). The composition of different whey proteins in bovine milk is given in Table 2.2.

Table 2.2 Approximate composition of whey proteins*

Whey protein	Proportion (% of cow's milk)
β-Lactoglobulin	0.32
α-Lactalbumin	0.12
BSA	0.04
Immunoglobulin	0.08
Lactoferrin	0.02
Lactoperoxidase	0.003
Enzymes	0.003
Proteose-peptones	0.1 – 0.3

*Adapted from De Wit (1998)

Among the whey proteins, the two most important are β-lactoglobulin comprising more than 50% of the total whey proteins in cows' milk and α-lactalbumin with 20%, while BSA and immunoglobulin correspond to around 6% and 12% of protein content of whey for each one (Fox and McSweeney, 1998).

The major whey protein, β-lactoglobulin (β-lg), is composed of globular molecules with a homogeneously distributed chain between polar and hydrophobic portions with a molecular mass (MM) of 18,277 Da. This protein is considered small if compared with other whey proteins such as serum albumin, enzymes, immunoglobulins and proteose-peptones (Eigel et al., 1984; Linden and Lorient, 1999). The milk of most ruminants and other species such as sow, dolphin or manatee contains β-lg, even if there are specific differences in β-lg of each species (Fox and McSweeney, 1998). There are seven genetic variants of β-lg that have been determined, however; only in five variants (A,B,C,D, and E) the

positions of amino acid susbstitutions have been established (Eigel et al., 1984). In bovine milk, there are four variants of β-lg; A,B,C, and D. Other types of β-lg have been found in milk from yak, ovine and cattle from Bali (Fox and McSweeney, 1998).

The primary structure of bovine β-lg consists of a sequence of 162 amino acids, including the 10 essential amino acids: Thr, Val, Met, Ile, Leu, Phe, Lys, His, Trp, and Arg (Castro and Avilia, 2003; Eigel et al., 1984; Fox and McSweeney, 1998). The secondary structure in the 2-6 pH range is formed by the folding of the polypeptide chain where monomers are joined to form dimers. It consists of approximately 10-17% α-helix, 24-43% β-sheet and 47% of not ordered structure (Lyster, 1972; Fox and McSweeney, 1998). The tertiary structure is compact and globular and the β-chains are combined in a barrel shaped structure giving monomers a spherical form (Fox and McSweeney, 1998).

The quaternary structure of bovine β-lg is strongly related to pH. Within pH range 5.5-7.5, the protein exists as paired molecules or dimers of 36,000 Da (Fox and McSweeney, 1998). At low temperature and below pH 3.5, these dimers separate to monomer structures of 18 kDa each, but in the range of pH 3.5-5.2, and mainly at pH 4.6, four dimers of bovine β-lg are linked to form octamers of 144,000 Da (Lyster, 1972; Aymard et al, 1996; Fox and McSweeney, 1998). Above pH 7.5, β-lg exists as unstable structure forming monomers containing thiol groups which produce sulphydryl-disulphide substitution reactions (Lyster, 1972; Aymard et al, 1996; Fox and McSweeney, 1998). Biological functions of β-lg are not well understood, but it may be deducted that the protein participates in the transport of vitamin A, owing to its ability to bind small hydrophobic molecules, and in the binding of fatty acids (Creamer and MacGibbon, 1996). On the other hand, cysteine, one of the rich sulphur

amino acids contained in bovine β-lg, reacts with the disulphide fractions of k-casein after heat denaturation affecting the rennet coagulation and being responsible for the "cooked flavour" of heated milk (Fox and McSweeney, 1998). Most of the precipitate produced by the heating of whey is due to β-lg, because of its predominant presence (higher than 50% of all whey proteins).

The second most abundant protein in bovine whey is α-lactalbumin (α-la), a globular protein with MM of 14,175 Da, also classified as a small protein owing to its small molecular mass. It possesses an excellent amino acid profile since is rich in essential amino acids and cystine (Fox and McSweeney, 1998). Its main biological function is to facilitate the enzymatic reaction between the substrate and galactosyltransferase for the synthesis of lactose (Yadav and Brew, 1991). Three genetic variants A, B, and C exist, but only the B variant is present in Western bovine cattle. The difference between A and B variants is that α-la A contains glutamic acid at position 10 while α-la B contains arginine at the same position in the amino-acid sequence (Eigel et al., 1984; Fox and McSweeney, 1998). The primary structure of the B variant consists of a chain of 123 amino acids, 10 of them essential (Castro and Avilia, 2003; Eigel et al., 1984). At pH range 5.4-9.0 α-la shows a stable conformation. The secondary structure consists of a coiled polypeptide chain composed approximately of 26% α-helix, 14% β-structure and 60% non specified structure (Lyster, 1972). The tertiary structure of α-la is still being studied, even if it is known that this structure is very close to the tertiary structure of lysozyme, while its quaternary structure is not yet known. This protein has the ability to bind Ca^{2+} resulting in it being the most heat resistant whey protein. It renatures after thermal denaturation and cooling at pH above 5, although a decrease in

the pH produces the loss of its ability to bind Ca^{2+} and the capacity to renature after heat treatment (Lyster, 1972; Fox and McSweeney, 1998).

Another important group of proteins in whey are Immunoglobulins (IG). These are a combination of glycoproteins with intricate structure and MM >146,000 Da which have a major immunological function, mainly in colostrums, although they also exist in milk, serum and other secretions. Immunoglobulins diverge considerably from other proteins contained in the serum due to their remarkable diversity, since they are generated as different polypeptide chains by a huge amount of cells. Consequently, most identification techniques usually applied to other proteins are mainly ineffective for IGs (Lyster, 1972; Eigel et al., 1984; Harper, 2000). There are five basic variants of IG in secretions of mammals: IGG, IGA, IGM, IGD, and IGE, although four of them (excluding IGD) have been found in bovine cattle milk. These proteins in monomeric or polymeric form comprise four amino acid chains (Eigel et al., 1984). The IGA molecule is formed by two heavy and two light chains of amino acids covalently bound. IGG exists as IGG_1, the main type, and IGG_2 whereas IGM is conformed by 2 light and 2 heavy chains connected in groups of five elements (Fox and McSweeney, 1998). The immunological activity of IG provides infection defense to newborns and adult individuals. The bovine calf obtains IG from the mother's milk the first days following the parturition (Harper, 2000; Fox and McSweeney, 1998).

Whey also contains a large protein called bovine serum albumin (BSA), with a molecular mass of 66,267 Da. It contains 582 amino acids, 17 disulphide links and 1 sulphydryl fraction. The amino-acid chain, like in β-lg and α-la, contains the 10 essential amino acids (Castro and Avilia, 2003; Eigel et al., 1984). The molecule comprises three elliptical structures, each of them forming 2 large and 1 short double loops. The biological function of BSA is the linkage of lipids and metals, having a

primary function in lipid oxidation (Eigel et al., 1984; Fox and McSweeney, 1998; Harper, 2000). It is also known that BSA may play a significant role in the synthesis of glutathione (De Wit, 1998).

2.2.2 Functionality of whey proteins

Whey proteins are not sedimented at the isoelectric point but have high sensitivity to heat due to their molecular structure. The irreversible denaturation of β-lg above 70°C is ruled by several factors: the time and rate of heating, the protein concentration, the pH, and the ionic strength. The highest sensitivity to heat denaturation of β-lactoglobulin is at pH values lower than 3.5 or over 7.5 since it exists as monomeric chains (De Wit, 1998; Sienkiewicz and Riedel, 1990). On the other hand, α-la at pH 4.5-6.5 is more resistant to the denaturation by heating than β-lactoglobulin due to disulphide bonds in its structure which permit a reversible thermal denaturation; however, under a strong heat process such as indirect UHT, α-la will be denatured irreversibly. BSA and IG are also denatured by heating temperatures over 70°C (Sienkiewicz and Riedel, 1990). If the concentration of whey proteins in solution is low, the result will be sediment formation. On the contrary, if the concentration is high enough, reversible or irreversible gels will be produced following thermal processing, as a result of the intensification of the interactions between the molecules leading to the reduction in the solubility (Aguilera, 1995). Formation of aggregates and their dynamics are characterized by the equilibrium of attractive and repulsive forces between denatured molecules of whey protein and the solvent. If a balance is reached the gel network will form. The charge of the protein molecules is related to the pH, while the ionic strength of the solvent commands the electrostatic interactions, translucent gels will be obtained when electrostatic attraction

is low (high repulsion), and solid and heterogeneous gels when the attraction is high (Aguilera, 1995; Lefebvre et al., 1998; Renard and Lefebvre, 1992).

The denaturation process has four stages; first the unfolding of the polypeptide chain occurs with a formation of irregular coils, which is an endothermic process and can be reversible or irreversible (Aguilera, 1995; De Wit, 1998). In this stage, the β-lactoglobulin structure is separated to monomer chains which can be linked to form reduced molecular mass aggregates, as results of the rupture of non-covalent bonds (Sienkiewicz and Riedel, 1990). Following the irreversible unfolding of the molecules, the forming of high molecular mass aggregates takes place associated with the presence of divalent calcium. The intramolecular reactions involved in the formation of aggregates are still unknown, but it is generally accepted that general interactions among unfolded molecular chains, hydrogen bonds, and ionic interactions participate as inducing forces (Aguilera, 1995; Sienkiewicz and Riedel, 1990). The third step is the formation of aggregates in a filament configuration, forming a consistent network of polypeptides and aggregates in a three-dimensional structure (Aguilera, 1995). The final stage is the association among the filament structures forming protein gels and networks. Whey protein gels seem to have a polymeric network with unlimited molecular weight in the gelling state (Aguilera, 1995).

2.3 Whey beverages

Whey has been used to produce alcoholic and non alcoholic beverages, such as whey beer and whey champagne, in several European countries and in the USA. Whey beverages have a nutritional role due to its high quality protein contribution, although in low

concentration, and its high content of calcium and other minerals such as sodium, potassium, and phosphorus (Jelen, 1992; Sienkiewicz and Riedel, 1990).

Traditionally, acid whey has been used in the production of beverages due to its low pH, high calcium content and large availability, although mixtures of rennet and acid whey are also employed as reported by Sienkiewicz and Riedel (1990). According to Prendergast (1985) whey beverages may be produced from fresh liquid whey (rennet or acid), dried whey (powder), and WPC and may include ingredients such as milk, preservatives, additives, buttermilk, yoghurt, bacteria cultures, and fruit juices. By the late 1980's, new developments in whey drinks were mainly based on mixtures of unprocessed or modified whey with fruit juices, and alternatively, beverages based in deproteinated whey and fermented or flavoured milk drinks containing whey or whey proteins (Jelen, 1992).

Whey-based drinks usually contain fruit juices or juice concentrates mixed with untreated whey which induces sedimentation and turbidity following heat treatment. Reduction of sedimentation can be achieved by centrifugation and a pH adjustement below 3.8-3.6, or 3.6-3.4 in the case of demineralized or sweet whey before UHT treatment. Turbidity caused by whey protein-pectin, whey protein-tannin or pectin-calcium interactions owing to the thermal processing of whey beverages, may be reduced by the addition of stabilizers (Jelen, 1992).

Whey beverages from fresh whole whey are cheap attractive alternatives because of the simply technology involved: fat separation, pasteurization, and degassing which resulted in low production costs (Sienkiewicz and Riedel, 1990). However, the proteins from fresh whey have the tendency to produce cloudiness and deposits, especially after a heating process at pH above 3.9, while fat may produce some atypical tastes. To avoid these quality problems, deproteinated and defatted whey

can be employed, which is still rich in minerals, vitamins and lactose (Jelen and Buchheim, 1984; Sienkiewicz and Riedel, 1990). Table 2.3 lists some European commercial whey and fruit-based whey beverages.

Table 2.3 Examples of whey-based and fruit-based whey beverages*

Name	description
Frusighurt (Germany)	Whey and 10% lemmon or apple
Big M (Germany)	Flavoured whey and vitamin E
Frucht-Molke (Heirler) (Germany)	Whey and apple, peach, passion fruit, or maracuya
Lattella (Austria)	Whey with mango, maracuya and grape/lime juices
Djoez (Holland)	Whey 80%, fruit juice concentrate, and flavour
Morea (France)	Whey concentrate and 40% mango, guava, kiwi, and passion fruit juices
Hedelmatarha (Finland)	Lactose-hydrolised whey and mango or tropical fruit juices
Rivella (Switzerland)	Clear deproteinated whey serum 35% and water
Surelli Fit (Switzerland)	Whey and 15% grape or mango juice
Fauna-fitt (Hungary)	Sweet UF permeate and mango, pineapple or strawberry juice

*Adapted from Jelen (1992)

Rolland (1999) described the production of a whey protein-orange juice drink by using enzymatically hydrolyzed whey proteins, which caused a noticeable reduction in the sedimentation following a severe heat treatment (100°C for 7 minutes). A secondary effect was the improvement of the nutritional properties, since hydrolyzed proteins were reduced to peptides with a MM < 5000 Da with better digestion.

Nowadays, some of the fruit-based whey and whey-based drinks that were very popular in the 80's such as Rivella, Big M, Frucht-Molke, Lattella, and Djoez are still in the market, according to current world wide web information, but facing the increasing demand for a new kind of protein beverages or "sport drinks" which include whey proteins or a mixture of whey, milk and other proteins.

2.4 Whey sport formulas

These products, also called "sport-drinks", can be produced as results of the development of new technologies such as ultrafiltration, reverse osmosis and electrodialysis, leading to the recovery of high purity proteins (WPI) in powder form.

Main types of whey protein powders include mixtures of whey protein hydrolysates, concentrates, and isolates blended with flavourings, natural colourings, artificial sweeteners, minerals, and vitamins. These products are mainly designed as protein supplement to fortify milk or fruit juices. Commercial examples of these products currently on the market are: Reflex Instant Whey, NOW Whey Protein, Veriuni Advanced Whey Protein, Aria, and Pro-Tein, the last one described as a "power-drink" and composed by a mixture of WPI, water and orange-pineapple flavour (Anonymous, 2006a).

Whey-based drinks produced from whole whey, mixtures of whey and fruit juices or fortified WPI drinks (sport formulas) contain whey proteins which are considered high quality functional proteins (Aoi, 2006). Recent reports (Anonymous, 2006b) indicate that in 2000 the world functional food market was estimated in 73 billion of euros, with a yearly growth of 16%. The European dairy sector alone showed an expansion of 6.8% per year.

Forming an important part of the European dairy sector, the dairy-based functional food market is considered as 3.9 billion euros, with Germany as one the most important consumers, since in 2004 a market report revealed that 34% of the population consumed whey drinks, while in 2006 the percentage increased to 50% (Anonymous, 2006b).

As shown in table 2.3, whey beverages are mostly composed by a mixture of whey and a variety of fruit juices. Pectin is a polysaccharide contained in the structure and peel of fruits, which may form complexes when mixed with whey proteins causing sedimentation, a quality defect that usually generates the major loss of acceptability from consumers.

2.5 Pectin

Pectin comprises the most complicated structure within the polysaccharides contained in cell walls, middle lamella and pulp of fruits and plants (Schols and Voragen, 2002). The vegetal wall polysaccharides can be classified in three major groups, based on structure and recovery methods; pectin, hemicellulose, and cellulose. These polymers can form complexes with proteins and phenols (Pilnik and Rombouts, 1985; Selvendran, 1985; Waldron et al., 2003).

Pectin is mainly composed of pectinic acids, such as galacturonic acid (GalA), containing rhamnose (Rha), arabinose (Ara), and Galactose

(Gal). It is distributed in the vegetal cell as part of a structural network containing cellulose and hemicellulose. The major functions of pectin are related to the properties of the cell wall: the protection against microorganisms, the ion transport, the water holding capacity and the regulation of the wall porosity (Steele et al., 1997; Bacic et al., 1988; Vincken et al., 2003).

2.5.1 Structure

The basic structure of pectin molecules is a linear chain formed by D-galactopyranosyl-uronic acid units connected by glycosidic bonds. The molecular backbone includes L-rhamnopyranosyl groups, lateral chains with neutral sugars, methanol and may also show limited acetylation according to BeMiller (1986). This author also stated that in pectin molecules, the proportion of monomeric units is not constant and the molecular weight varies depending on the different fruits and the processing treatments, resulting in obtaining a variety of pectin types. In natural pectin, some carboxyl fractions are esterified to the methyl ester form while the rest may be associated with sodium, potassium or ammonium carboxylate. BeMiller (1986) affirmed that pectin can have a different amount of carboxyl fractions containing methanol or also denominated degree of esterification (DE). Pectin with DE higher than 50% is called high-methoxy pectin or HM-pectin; when the DE is lower than 50% the product is denoted as low-methoxy pectin or LM-pectin. The proportion of carboxyl fractions in the amide form is called the degree of amidation or DA. The DE is significantly important because it rules the main functional properties of pectin such as solubility, gelation capacity, gelling temperature and the characteristics of the formed gels.

Ralet (2004) declared that some pectin molecules can have acetates units associated to O-2 or O-3 positions in the D-galactopyranosyluronic acid molecules causing the complete restrain of the gelation. The sugar-beet pectin chains are partially methylated and acetylated, and its limited gelling capacity is because of the presence of acetyl groups which esterify the galacturonic acid fractions as illustrated in Fig. 2.1. Some other pectins similarly esterified by acetyl groups are obtained from sunflower and potato (BeMiller, 1986).

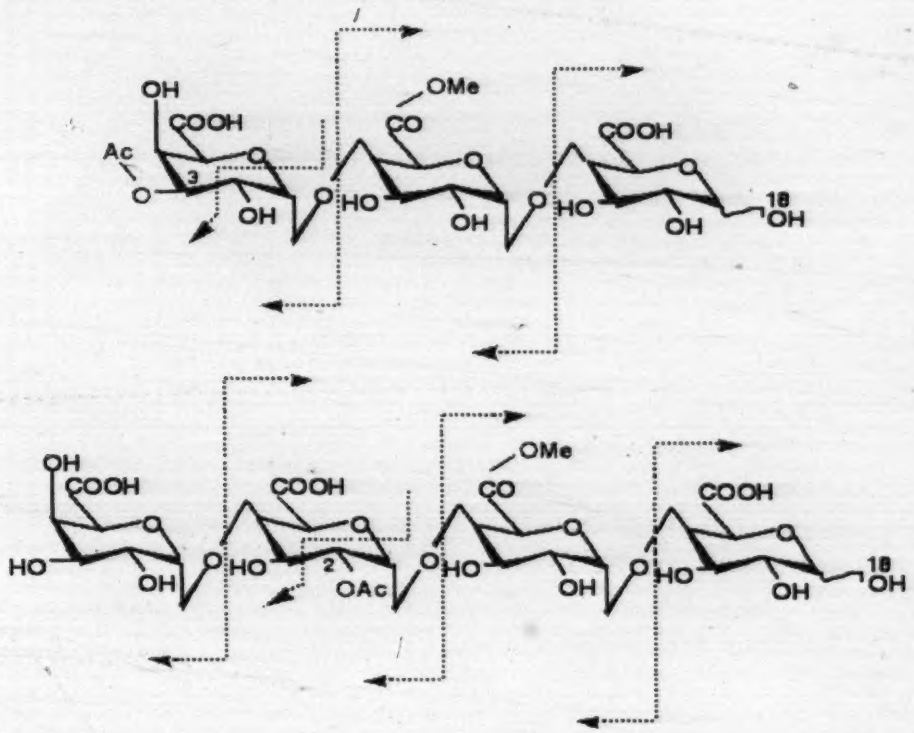

Fig 2.1: Partly acetylated and methylated units of sugar-beet pectin by acetyl groups on position O-3 and O-2 (adapted from Ralet, 2004).

Further polysaccharides of pectin molecules are arabinans, galactans, arabinogalactans associated to rhamnogalacturonan fractions, and xylogalacturonan, one of the forms of galacturonan in the wall structure, while protopectin is the insoluble pectin fraction which is extracted by heating and acid treatments (Schols and Voragen, 1994; Vincken et al., 2003; Schols and Voragen, 2002).

The main constituents of commercial pectins are homogalacturonans (HG). These components vary by the level of methyl ester and the distribution of these esters over the structure, where non-esterified galacturonic acid fractions and methylated portions are mixed (Schols and Voragen, 2002). Portions of galacturonic acid units may also be replaced by acetyl groups depending on the source of the pectin (Daas et al., 2000 a,b).

Pectin contains also rhamnogalacturonan (RG), consisting of rhamnose and galacturonic-acid fractions of pectin units. These are intricate structures having only the rhamnogalacturonan portion as the common component (Schols and Voragen, 1994; Schols and Voragen, 2002), while rhamnogalacturonan II (RG-II) is a structural polysaccharide with a scarce presence in cell walls (Waldron et al., 2003). The probable function of RG-II is the linkage of pectin molecules inside the wall (Pellerin et al., 1996; Vidal et al., 2000). Other constituents of pectin are arabinans, large polysaccharide molecules composed of a main structure and single side chains of α–Ara, and arabinogalactans which are polysaccharides consisting of a β–Gal structure with lateral-chains of α–Ara (Waldron et al., 2003). Xylogalacturonans (XGA) are formed by linear sucessions of homogalacturonan with a single fraction of xylose attached to the galacturonan molecule (Vincken et al., 2003; Schols and Voragen, 1994). Galactans are mainly linear sequences of (1-4) or (1-6) carbon linked β–Gal molecules (Waldron et al., 2003).

Pectin molecules and HG, XGA, RG, RG-II, β-galactan, α-arabinan, and arabinogalactans are associated by covalent bonds. The interactions between pectin and other macromolecules provide the nature of the plant wall structure (Schols and Voragen, 2002).

2.5.2 Functional properties

The high significance of pectin as a food ingredient is because of its gelling and thickening capability. Pectin combined with other carbohydrates produces gels in acid aqueous solutions (Leroux et al., 2003). The gelation takes place following interactions among the dissolved macromolecules to form a network where solvent and solute are braided, however, to form a gel the new bound portions must be of a small size, otherwise, a precipitate and not a gel will be produced. The units of L-rhamnopyranosyl attached to the pectin structure confer the irregularities ("kinks") which restrict the size of the bound chains (BeMiller, 1986). When acid is added to pectin, the negative charges of the carboxylated fractions are eliminated producing new linkages among the pectin chains and a reduced hydration of the molecules, which are associated to calcium (BeMiller, 1986). In addition to the concentration of pectin, the concentration of co-solutes, like sugar, and the proportion of cations are the main factors involved in the gelation process, while the characteristics of the gel are pH and temperature dependent. At moderate temperature (4°C) and pH lower than 3, esters are removed resulting in the hydrolyzation of the sugar fractions, however, at alkaline pH (about 8), ester and sugar linkages can be also broken easily resulting in the releasing of methyl ester groups at an escalating rate if the pH is also increased, causing slowness or inhibition of gelation (Pilgrim et al., 1991; Schols and Voragen, 2002).

The gelation of HM pectins is caused by a combination of polymers in water solution and in an acidic condition, pH 2.9-3.2, in association with carbohydrates or other co-solutes. HM pectin gels will change in an irreversible way regarding changes in the temperature (Hoefler, 1991; Schols and Voragen, 2002).

The gelation of LM pectins will happen because of the reaction with calcium or other cations, since calcium links free carboxyl groups on pectin and connects them, creating a network which contributes to the rigidity of the structure. LM pectin produces gels that may be reversible depending on the thermal treatment (Barret et al., 1998; Hoefler, 1991; Schols and Voragen, 2002).

Other associated parameters in the gelation ability of pectin are the DA, the DE, the molecular weight, and the occurrence of acetyl fractions. HM, LM and low-methoxylated amide (LMA) pectins have diverse functionality degrees although the molecular weight, methylation level, sugar and galacturonic acid contents are comparable. Optimizing the specific commercial applications of pectins is difficult due to this characteristic (BeMiller, 1986; Schols and Voragen, 2002).

An unusual attribute for a gel forming material, the emulsifying property, is not related to the chemical structure of pectin according to Dea and Madden (1986). The emulsifying ability of citrus pectin and beet pectin in oil emulsions was studied by Leroux et al. (2003); they concluded that pectin can stabilize oil in water mixtures with the same quality as gum arabic although in a much lower concentration, and that beet pectin has better emulsifying capacity than other sources of pectin such as apple and citrus. They also stated that the acetyl groups of pectin associated with protein molecules generate stable emulsions.

2.5.3 Natural pectin *vs.* prepared pectin

When fruits are mashed for juice production, the pulp and the liquid obtained contain pectin. In the case of apples, the juice produced is cloudy and has a tendency to form sediment after a short time. The cloudiness is produced by some polysaccharides such as pectin, cellulose, starch and other substances like proteins, minerals, and microorganisms (Alvarez et al., 1998). Pectin, starch and other colloidal particles in suspension will also produce major problems during filtration and clarification if they are not degraded by a specific enzymatic treatment (Pilnik and Rombouts, 1985; Alvarez et al., 1998).

Pectin is isolated from pomace or peel of apple and lemon. An alternative limited source of pectin is the sugar beet pulp. All of these materials are by-products of the agricultural industry (Leroux et al., 2003). Industrial pectin differs from natural pectin since its functionality can be modified as required by the extracting and refining process (Schols and Voragen, 2002). Industrial pectin contains at least 75% of galacturonic acid with a degree of methylation up to 80%. When used in food applications, its fluidity and viscosity characteristics are mainly related to amidated pectin containing an NH_2 instead of an OCH_3 group (May, 2000).

The first step in the processing of pectin is the washing of the peel, pulp, and other residues of fruits. Some citrus fruits like lemon and lime produce pectins with low calcium compatibility and with high strength and viscosity, while oranges and grapefruit generate pectins with lower viscosity and higher calcium sensitivity, making them inappropriate for gel development in foods with calcium rich components, as informed by Rolin (2002).

In the second step, the washed fruit parts are plunged in a hot water bath at 90°C containing strong nitric acid concentration (pH 1-3) and filtered after several hours. Then the insoluble solids are isolated from the solution, concentrated and mixed with alcohol to produce the sedimentation of pectin. The precipitated pectin is purified by consecutive alcohol washing and then dehydrated and grinded (Rolin, 2002).

Protopectin fraction, the water insoluble precursor of pectin, is also turned into soluble pectin after this operation, probably because of the hydrolysis of its molecular components. The DE of pectin and the degree of hydrolysis are ruled by variations on the pH and temperature, consequently, the functionality of pectin can be modified depending on the isolation techniques and pectin sources. The by-products of the pectin processing are usually used as forage (Rolin, 2002). A de-esterification stage following filtration produces low DE pectin. The hydrolyzation can be made by using NaOH at low temperature and with pectin not in water solution, to prevent undesirable reactions because of the susceptibility of the pectin structure in alkaline media. Amidated pectin is obtained if ammonia is employed as alkaline agent instead of NaOH. In a final step, industrial pectin must be standardized according to established specifications including DE, maximum proportion of contaminants, sugar content and strength degree. Food degree pectin must comply with FAO, Food Chemicals Codex and other USA specifications, while pectin for pharmaceutical purpose must be in agreement with the specifications of the US Pharmacopeia and National Foundry (Rolin, 2002).

2.5.4 Pectin in beverages and solids foods

Although pectin in the production of high viscosity foods is a functional ingredient, its main contribution in terms of quality as a food

polysaccharide is restricted to the fiber supply (Pilnik and Rombouts, 1985). The processing of fruits for juice production may vary widely depending on the character of the final product desired, cloudy or clear. In the cloudy juice, there is no clarification, decanting or filtration; the main characteristic of this juice is the turbidity. Hence the presence of pectin and other carbohydrates in the solution is highly desirable. On the contrary, the property of pectin as a gelling, stabilizing or turbidity forming agent is extremely undesirable in clear juice; thus, enzymatic treatments must be applied (Pilnik and Rombouts, 1985).

Pectinases in addition to amylase enzymes are used to hydrolyze and flocculate pectins and starch; this results in a juice with less viscosity and lower amount of colloid carbohydrates which can be easily filtered or ultrafiltered (Alvarez et al., 1998). Yu and Lencki (2004) used two types of apple to study the effects of the enzymatic degradation in the clarification of apple juice. McIntosh juice without treatment had lower flux than enzymatically treated Red Delicious juice. However, subsequent addition of polygalacturonase (PG) and pectin lyase (PL) into the McIntosh juice produced an increase in the flow of permeate similar as Red Delicious. On the contrary, when pectin esterase (PE) was added to McIntosh or Red Delicious juice, it resulted in higher flux resistance owing to a fouling coat with low porosity having been produced. Enzymatic treatment with PG and PL increased the interactions among colloidal particles, producing higher aggregation and high porosity. The best result was obtained by a mixture of PG or PL with PE, resulting in a gel coat with the right degree of porosity (Yu and Lencki, 2004).

Klahorst (2002) claimed that in some kind of drinks, the viscosity provided by pectin is a highly desirable trait because of the texture feeling. Additionally, the viscosity also confers stability to the suspension by limiting the precipitation of macromolecules such as sugars and

proteins. In cloudy juices mixtures of pectin, gum Arabic, or other gums provide the desired level of viscosity. Klahorst (2002) also stated that the complexes developed at low pH (3.7-4.3) between pectin and casein give the adequate viscosity to fruit juices and yoghurt, whereas pectin and maltodextrin are generally used to stabilize acidified milk. According to BeMiller (1986) the most common utilization of industrial pectin in food industry is not in beverages but in the manufacture of jellies, jams, marmalades and confectionary products, all of them foods with reduced water activity.

2.6. Interactions of proteins with pectin

2.6.1. General mechanism of interactions between proteins and polysaccharides.

The complexation between proteins and polysaccharides has been stated to be the result of ionic interactions between molecules with an opposite electric charge; thus, protein-polysaccharide interactions are highly pH-dependent but also influenced by the ionic strength, molecular structure and concentration of polymers (Hidalgo and Hansen, 1970; Dickinson, 1998). Soluble complexes between anionic polysaccharides and proteins will be produced when the net charge of both biopolymers is negative, above the isoelectric point of the protein, although positively charged local portions of protein interact with the polysaccharide. If the negative charge of the protein is increased, the protein-protein repulsion is increased and the protein-polysaccharide interaction is reduced (Dickinson, 1998). Interactions between proteins and polysaccharides at pH values drastically below the isoelectric point of the protein may lead to

the formation of insoluble complexes with highly opposite electric charge between both molecules (Dickinson, 1998).

At low ionic strength and neutral or alkaline pH, some polysaccharides such as carrageenan and dextran form strong reversible complexes with proteins, since the attraction of NH_3^+ fractions of protein for OSO_3^- fractions is stronger than for CO_2^- molecules (Dickinson, 1998). A complex composed by κ-casein-carrageenan in some dairy products is a descriptive example. On the contrary, carboxylated LM pectin and casein do not undergo complexation at neutral pH (Imeson et al., 1977; Dickinson, 1998). Thus, anionic polysaccharides such as pectate, alginate, and carboxymethylcellulose (CMC) interact with proteins mainly as a result of pH and ionic strength variations. Whey proteins such as β-lg, α-la, and BSA formed complexes with CMC at pH 4 which is below their IEP, 4.7 for α-la, 5.2 for β-lg, and 4.9 for BSA, with decreased solubility compared to the solubility of the whey proteins alone. The complexation between whey proteins and CMC only takes place at ionic strength lower than 0.2. (Hidalgo and Hansen, 1970; Hill and Zadow, 1978; Conrado et al., 2005).

2.6.2. Whey protein-pectin interactions

Whey proteins and pectin can form protein-pectin complexes because of non covalent bonds. In this case the linkage is weak and reversible by changes in pH or temperature. Alternatively, covalent bonds can be also formed and in this case a protein-polysaccharide molecule with stable linkage will be the result (Dickinson and Galazka, 1991; Neirynck et al., 2004).

Pectin and BSA form complexes with reversible changes in the viscosity depending on the pH (Takada and Nelson; 1983). An unheated

system formed by pectate and BSA at pH 6 and ionic strength 0.025 shows very weak interactions owing to the net charge of the protein at such pH being negative; however, after heating, high molecular mass stable complexes (MM>100.000) are formed between both polymers. The pectate produced the inhibition of the protein-protein aggregation in BSA and its precipitation (Imeson et al., 1977). It is suggested that the electrostatic interactions are produced between carboxylated groups of pectin and ε-amino, α-amino, guanidinium and imidazole sites of the protein; thus, the number and distribution of these portions and the net charge of the protein, are relevant factors in the strength of the interactions. The heat denaturation of the protein produces the unfolding of the coiled molecule and liberation of "buried" groups containing these sites, which cause increasing protein-pectin interactions forming more stable complexes (Imeson et al. 1977; De Wit, 1998).

High methoxy (HM) and low methoxy (LM) pectin undergo interactions with β-lg at pH 4.5 and weak ionic strength condition. Systems with protein/pectin ratio 4:1 showed that approximately 96% of β-lg conjugated with LM pectin, while only 78% of β-lg formed a complex with HM pectin, as a consequence of the inferior amount of carboxylic groups in HM than in LM pectin. Heat treatment decreased the HM pectin-β-lg complexation but in contrast, increased the interactions between LM pectin and β-lg (Girard et al., 2002).

Mixtures of orange juice and α-la, β-lg, or WPI in pH and temperature ranges 3.0-5.0 and 65°C-85°C, showed that the transmittance (%T) increased with increasing pH and decreased when the temperature rose. Analysis of the supernatant indicated wider juice-protein interactions for β-lg and WPI than for α-la, particularly at low pH. Thus, β-lg and WPI induced the formation of more charged soluble

aggregates at low pH and high temperature than α-la (Kazmierski et al., 2002).

The complex formed by whey proteins and pectin was used in the isolation of whey proteins from whey by Serov et al. (1985). The maximum yield of proteins in the insoluble complex was observed at ionic strength 0.01 and pH 3.4; however, traces of α-la were also found in the diluted phase of the system. At constant protein content, the amount of protein in the complex depended on the pH and concentration of pectin in the system.

Whey protein-pectin complexes show easier formation than casein-pectin conjugates, due to the higher volume exclusion of casein molecules and their more compact structure, which impede stronger interactions between casein and pectin (Einhorn-Stoll et al, 2004). The addition of LM pectin in a range 0.5%-1.5% to 8% whey protein solutions at pH 6 produced gel formation. A subsequent addition of calcium up to 10mM concentration increased the firmness of the gel, although gels produced by a mixture of 10mM calcium with whey proteins alone (without pectin) were stronger than the gel formed in the protein-LM pectin-calcium system. The competition between LM pectin and proteins for Ca^{2+} leads to a less amount of Ca^{2+} available for the proteins (Beaulieu et al., 2001). The degree of methylation and the protein/pectin ratio affects the solubility of a whey protein-pectin complex. For LM pectin the minimal protein solubility is at a protein/pectin ratio 4:1 while for HM pectin the protein/pectin ratio is 2:1(Neirynck et al., 2004).

2.6.3. Interactions between pectin and other proteins

Mort (2002) stated that pectin and protein complexation is the result not only of ionic interactions between acidic groups of pectins and

basic groups of proteins, but probably due to direct amide linkages between pectins and proteins as well. The covalent linkage between acetyl groups of pectin and proteins such as hydroxyproline, extensin, and kinases, is mentioned as an example of natural pectin-protein complexation (Leroux et al., 2003; Mort, 2002).

Imeson et al. (1977) illustrated the electrostatic nature of the interactions among sodium pectate, alginate, and CMC with myoglobin. Their results confirmed the dependence of the complexation on the pH and ionic strength, since the interactions in all systems increased when the pH diminished from 7 to 5, but decreased when the ionic strength increased.

In industrial applications, pectin is used to increase the viscosity of yoghurt, since at pH around 4 (below the isoelectric point of casein) the positive charged proteins bind the anionic pectin forming a pectin-casein network with increased viscosity. Pectin is also employed as a stabilizer in cultured milk beverages such as drinkable yoghurt, owing to its anti-coagulation properties in the presence of milk proteins (Foley and Mulcahy, 1989: Rolin, 2002). In acid-milk drinks, HM pectin has a higher stabilizing effect than LM pectin, since its lower amount of carboxyl groups sustain feebler interactions with milk proteins than LM pectin. As a result, a shorter portion of HM pectin interacts with the casein molecule and a higher fraction of pectin is free to interact with the solvent, producing a more stable dispersion (Dickinson, 1998).

The abundant information about the application of pectin as viscosity provider and stabilizer agent in yoghurt and other high viscosity foods, contrasts with the scarce research reported on the effects caused by the occurrence of pectin in fruit juice and whey-based drinks. In the case of tomato products fortified with whey concentrate specifically, the

available studies about the interactions between tomato pectin and whey proteins are almost non existent. Thus it was decided to investigate in a closer view some effects of the interactions between whey proteins and pectin in WPI-tomato juice mixtures, as a preliminary phase to the potential formulation of a successful commercial tomato juice beverage containing WPI.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Whey protein isolate

Commercial whey protein isolate (WPI) in powder form was used for all the experiments. It was supplied by Land O' Lakes, Inc, Arden Hills, MN. It contained 90% protein on a dry basis, with less than 1% lactose and 0.20% fat, as specified in the technical data sheet.

3.1.2 Cottage cheese whey

Acid cottage cheese whey (CCW) was prepared in the laboratory by adding concentrated lactic acid (85% v/v) to skim milk purchased locally. The milk was acidified to pH 4.7 at 35°C. The mixture was stirred by a magnetic stirrer/heater, "IKA-Combimag RET" (IKA-WERK, Staufen, Germany), for 10 minutes at 200 rpm. The whey obtained was decanted for 6 hours at 20°C and then separated from the coagulated casein, vacuum filtered, and stored at 2.5°C.

3.1.3 High methoxy (HM) pectin

High methoxy (HM) pectin, P-8471 produced from apples with a degree of esterification of 74% as declared by the manufacturer, was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.1.4 Tomato juice

Commercial tomato juice, produced from the processing of fresh tomatoes and not from concentrate as declared by the manufacturer, H.J. Heinz Company of Canada Ltd., North York, Ontario, was obtained locally. The product was supplied in 1.36 L cans.

3.1.5 Polygalacturonase enzyme

Pectinase enzyme Sigma P-2401 in crude powder form, produced from Rhizopus sp. (polygalacturonase), was supplied by Sigma-Aldrich, St. Louis, MO, USA. According to producer's specification one unit (0.002 g) of the enzyme liberates 1.0 µmole of galacturonic acid per minute at pH 4.0 and 25°C.

3.1.6 Chemicals for analyses

Chemical products used for pH adjustments and for pectin analyses, including lactic acid, sulphuric acid, sodium tetraborate, and galacturonic acid, were obtained from Sigma-Aldrich , St. Louis, MO, USA.

3.2 Experimental methods

3.2.1 Preparation of whey protein solutions. First model system

Sets of 100 mL solutions containing 2%, 4%, or 6% w/w of WPI powder in deionized water were prepared in separate beakers. The pH

was adjusted to 3.3, 3.7 or 4.2 through the addition of concentrated lactic acid (85% v/v).

The solutions were gently stirred using manual and magnetic stirring for 1 hour and left in graduated cylinders for 18 hours at 20°C to allow for the complete hydrating of the WPI, and estimation of the insolubility.

Heated solutions were prepared by heat treatment of the solutions in a hot water bath at 92°C for 15 minutes. The time to reach 92°C, 14 minutes, was not included in the heating period. After heating, the samples were immediately cooled to 20°C in an ice water bath and kept at this temperature for 6 hours. Visual sedimentation was determined in all samples.

3.2.2 Yield of sediments

Unheated and heated mixtures containing different proportions of WPI and deionized water at the three pH levels, were prepared as described in the Section 3.2.1. Then, 100 mL aliquots of each mixture were centrifuged at 550 x g for 30 minutes in a 6 tube rotor "MSE" bench table-top centrifuge (MSE, Crawley, Sussex, U.K.).

After centrifugation, the supernatant was separated from the wet sediment. The wet sediment was washed with 100 mL of deionized water and the mixture dried at 70°C (AOAC, 2003) for 15 hours or until a constant weight was obtained in a "National Appliance 5851" vacuum oven (National Appliance Co., Skokie, IL, USA). The obtained dry sediment was weighed in an electric "Mettler PE 3600" balance (Mettler Instrument AG, Zurich, Switzerland).

3.2.3 Pectin-protein interactions. Second model system

Solutions containing 1% WPI and 0.10%, 0.20%, or 0.30% w/w of high methoxy (HM) pectin were prepared by direct addition of pectin in powder form into the solutions. The resulting pH levels of the blends were 5.6 for the solution containing 0.10% pectin, 5.3 for 0.20% pectin and 5.0 for 0.30% pectin respectively. Three solutions at pH values 3.3, 3.7 and 4.2 were then prepared for each concentration of pectin by adjustment of the pH with concentrated lactic acid. The solutions were stirred for 1 hour at 20°C and kept at 20°C for 18 hours. Then 100 mL batches were centrifuged at 550 x g for 30 minutes.

After the wet sediment was separated from the supernatant, the wet sediment was weighed, washed, and dried as described in the Section 3.2.2. The dry sediment was weighed to estimate the yield while the supernatant was discarded.

Measurements of the effect of temperature on protein-pectin interactions were made after keeping the prepared solutions at 20°C for 18 hours, then heating them at 92°C for 15 minutes, cooling them to 20°C, and centrifuging at 550 x g for 30 minutes. After the wet sediment was separated from the supernatant, the wet sediment was dried under vacuum conditions at 70°C for 15 hours and weighed.

3.2.4 Whey protein-pectin interactions in tomato juice-WPI mixtures. Third model system

Mixtures containing 2%, 4%, 6%, 8%, 10%, 12% or 15% w/w of WPI in tomato juice were prepared by mixing WPI powder into the tomato juice. Manual and magnetic stirring at 200 rpm were applied to all mixtures for at least 1 hour or until the WPI was completely

solubilized in the tomato juice. The pH was adjusted to 3.3, 3.7, or 4.2 by the addition of concentrated lactic acid (85% v/v) and the mixtures were left for 18 hours at 20°C. Heating, cooling and centrifuging the WPI-tomato solutions were carried out as described in Sections 3.2.1 and 3.2.2.

3.2.5 Viscosity

The viscosity of the WPI-tomato juice mixtures was determined by using a rotary "Brookfield RVTD" digital viscometer (Brookfield, Engineering Laboratories, Inc. Stoughton, MA, USA) with spindle RV3 at 100 rpm. All determinations were made at 20°C, employing the same N°1000-150 mL beaker. Aliquots of 140 mL of each solution were placed in the beaker with the spindle submerged in the mixture until the notch was fully covered. The viscosity was continuously measured for 720 seconds (12 minutes) or until the value in the digital display was stabilized. Each measurement was made at least in duplicate.

3.2.6 Enzymatic hydrolysis of pectin in tomato juice

In order to study the effect of the breakdown of the pectin present in the tomato juice by an enzyme, the viscosity and pectin content of the sediments of enzymatically treated tomato juice were measured.

Aliquots of 150 mL of tomato juice were prepared in separate beakers, and different percentages of polygalacturonase enzyme (0.05%, 0.10%, 0.15%, 0.30% or 0.50%) were added to the aliquots. The pH was adjusted to 4.2, which is very close to the natural pH of the tomato juice (4.21-4.25), by using concentrated lactic acid (85% v/v). The mixtures were gently mixed by magnetic stirring for 1 hour at 20°C.

After this time, the samples were placed in a hot water bath at 40°C and left for 18 hours at that temperature. Following cooling to 20°C, measurements of viscosity, yield of sediment, and pectin content of sediments were carried out for each sample.

3.2.7 Enzymatic hydrolysis of pectin in WPI-tomato juice mixtures

Polygalacturonase enzyme in 0.05%, 0.10%, 0.15%, 0.30% or 0.50% concentration was added to 144 mL aliquots of tomato juice in separate beakers. The pH adjusting, mixing, heating in a hot water bath and cooling were carried out as described in the section 3.2.6. The next stage was the addition of 6 g of WPI to obtain a mixture with 4% WPI and enzymatically treated tomato juice. The mixtures were manually and magnetically stirred for 1 hour and left at 20°C for 18 hours. After that time the viscosity, pectin content and yield of sediment were analyzed.

3.2.8 Whey protein-pectin interactions in tomato juice-CCW mixtures

CCW and tomato juice were mixed in 50-50% proportion to produce CCW-tomato juice blends. Adjustments of pH to 3.3 and 4.2 were made through the addition of concentrated lactic acid. Mixtures were stirred at 200 rpm for 1 hour and left for 18 hours at 20°C as in the case of the WPI-tomato juice blends. Heating, cooling, and centrifuging the CCW-tomato juice samples were accomplished as described in Sections 3.2.1 and 3.2.2. Measurements of viscosity were carried out as described in Section 3.2.5.

3.2.9 Spray drying of WPI and CCW-tomato juice mixtures

Batches containing 300 mL of mixtures 4% WPI and 96% tomato juice were prepared by mixing WPI powder directly into the tomato juice. The solutions were stirred until the powder was completely dissolved and then the pH was adjusted to 3.3 and 4.2. The solutions were left for 18 hours at 20°C.

Mixtures containing 48% CCW and 48% tomato juice were produced by mixing both components and stirring for 1 hour. Then, when required, 4% of WPI was added to the mixtures and stirred until complete dissolution of the powder. Batches of 300 mL of these mixtures were prepared at pH 3.3 and 4.2 and kept at 20°C for 18 hours. After this time, the WPI-tomato juice and CCW-tomato juice-WPI mixtures were spray dried employing a Buchi "Model 190" co-current mini spray drier (Buchi Labortechnik AG, Flawil, Switzerland). The blends were pumped by a peristaltic pump using a single 0.5 mm nozzle at a feeding rate of 5 g/min, keeping the inlet and outlet air temperatures at 130°C and 63°C respectively. The outlet air temperature was maintained by controlling the feeding rate of the product. The air aspirator capacity was approximately 22.2 kg air h^{-1}. The dried powder samples recovered from the powder collector and body of the cyclone were kept in sealed beakers and stored at 2.5°C for determinations of solubility, sedimentation behaviour, and moisture analysis.

3.3 Analytical Methods

3.3.1 Total solids

The total solids contents of the mixtures were determined through

the use of a "Samsung MW5580W" microwave oven (Samsung Electronics Inc., Sukwon, Korea). Aliquots of 0.5 g of each sample were placed into pretared glass cups with a filter paper and weighed. After drying in the microwave oven set at a power of 6 for 15 minutes, the samples were weighed again. The dry weight and total solids were estimated by determining the difference between both weights.

3.3.2 Measurement of pH

The pH was determined by using an "Orion-Research mod. 701-A" digital ionalyzer pHmeter (Orion-Research, Florida, MI, USA). All determinations were made at least in duplicate at 20°C.

3.3.3 Pectin content

Pectin content was determined according to a colorimetric assay adapted by Kintner and Van Buren (1982) from the method used by Blumenkrantz and Asboe-Hansen (1973). Samples (1mL) and separate aliquots (1mL) of galacturonic acid standard solutions were diluted by the use of distilled water. The new solutions containing uronic acid in a concentration ranged between 5 and 75 µg /mL were placed into separate test tubes and cooled in an ice water bath. After some minutes, 6 mL of sulphuric acid-tetraborate solution were added to each test tube in water bath and mixed using a "Vortex-Genie 550-6" vortex (Scientific Industries Inc., Bohemia, MA, USA). Then the test tubes were heated in boiling water for 5 minutes and cooled in the ice water bath.

Following cooling for some minutes, 0.1 mL of 0.15% m-hydroxydiphenyl solution was added to the blend in each test tube and vortexed again. Solutions developed a soft red color and were allowed

to stand for 20 minutes in order to dissolve air bubbles. Then the absorbance of each solution was read in duplicate as absorbance index (AI) at 520 nm, by using a "Spectronic 21" spectrophotometer (Baush & Lomb Co. Rochester, NY, USA). Sample blanks were run for each sample solution, because a red color is also produced by carbohydrates heated in sulphuric acid and tetraborate; however, in sample blanks the m-hydroxydiphenyl was replaced by 0.1 mL of 0.5% w/v sodium hydroxide, keeping the rest of the procedure exactly the same. The absorbance values of sample blanks were subtracted from the total absorbance of solutions to obtain the absorbance due to the presence of m-hydroxydiphenyl.

The absorbance values read by the spectrophotometer were compared to a previously constructed standard curve, showed in Appendix 1, made with different concentrations of galacturonic acid standard solutions ranging from 0 to 80 µg/mL. For the calibration of the spectrophotometer a reagent blank solution was prepared containing 1mL of distilled water, 6 mL sulphuric acid and tetraborate solution, and 0.1 mL 0.5% sodium hydroxide.

3.3.4 Protein content

The protein determinations were made in duplicate by means of an automatic "LECO FP-428" nitrogen/protein determinator (Leco Corporation, St. Joseph, MI, USA). The factor used for the nitrogen content was N x 6.38 (AOAC, 2003).

3.3.5 Determination of visual sediment

Samples of 100 mL solutions of 2%, 4%, or 6% WPI in deionized

water and 1% WPI mixed with 0.10%, 0.20%, or 0.30% HM pectin were prepared in separate beakers and the pH adjusted to 3.3, 3.7, or 4.2. The solutions were left standing in separate 100 mL graduate cylinders for 18 hours at 20°C after which the volume of the sediment formed, sometimes seen as a "cloud", was determined by visual observation.

3.3.6 Moisture content

The moisture content of spray dried powders was measured by drying the samples in powder form through the use of a vacuum oven at 70°C for 15 hours or until a constant weight was obtained as described in section 3.2.2. The moisture contents of samples were determined by the difference between the weight of the powder before and after drying.

3.3.7 Solubility

The solubility of the spray dried mixtures was determined by a method adapted from El-Tinay and Ismail (1985). A 2 g sample in powder form was added to 50 mL of distilled water at 20°C under continuing stirring. The blend was agitated in a 100 mL beaker using a magnetic stirrer at 100 rpm. The time needed for the visual dissolution of the powder in the water was measured by means of a chronometer.

3.3.8 Statistical Methods

Experiments were at least replicated, and analyses were carried out at least in duplicate generating n = 4 or more. Results are expressed as a mean $\pm$ standard error of the mean (S.E.M.). Statistical significance of differences ($P < 0.05$) was determined by Anova univariate, Anova

one way, and Paired T-test using the Statistical Package for the Social Science (SPSS 14.0) for window program. Regression analysis was also used when necessary.

4. RESULTS AND DISCUSSION

The behavior of whey proteins in aqueous systems was first studied by preparing solutions of WPI and deionized water as the first model system to serve as baseline for the subsequent studies. Then, the interactions between whey proteins and pectin were investigated in solutions of WPI and pectin as the second model system and in mixtures of WPI and tomato juice as the third model system.

4.1 Whey proteins in aqueous system

4.1.1 Unheated WPI solutions

No visual sedimentation was obtained after mixing WPI in 2, 4, or 6% concentration with deionized water at pH 3.3 and 3.7, while visual sedimentation was noticeable in the same solutions at pH 4.2. Figure 4.1 shows the amount of visual sediment observed from 100 mL of unheated blends with different concentrations of WPI at pH 4.2. The amount of sediment ranging from 3 to 11.1% increased with the increasing concentration of WPI in the solutions: i.e., the lowest amount of sediment was observed at 2% and the highest at 6% WPI. Although the native whey proteins do not precipitate at their isoelectric point, the visual sedimentation noted at pH 4.2 indicates that some small amount of insoluble proteins was present in the WPI used, possibly as a result of thermal damage during drying. Also, Burgess and Kelly (1979) found that in extremely weak salt concentrations, β-lactoglobulin and immunoglobulins have unstable solubility characteristics, which results in protein deposition that can be reverted by increasing the ionic strength or increasing the pH up to 7.0. Jelen and Buchheim (1984) observed

turbidity development in unheated acid whey samples in the pH 4.8-2.5 range. After the solutions were centrifuged, different volumes of sediments were obtained at all WPI concentrations and pH levels as shown in Fig. 4.2. Thus, the centrifugation may cause the sedimentation of slack aggregates in suspension that are not visible in the whey solutions at pH 3.3 or 3.7. The quantity of dry sediments increased with the increasing amount of WPI added at the same pH. Significant differences ($p<0.05$) after statistical analysis were obtained in the amount of sediment from solutions with the same concentration (2, 4, or 6%) of WPI, but different pH. The amount of dry sediments produced at pH 3.3 was the lowest, followed by 3.7 and 4.2.

4.1.2 Heated WPI solutions

Heating of 2, 4, or 6% WPI solutions resulted in no visual sedimentation produced at pH 3.3 and 3.7, but again, a significant amount of sediment was observed at pH 4.2. The amount of sediment produced in 100 mL of heated solutions at pH 4.2 was significantly higher ($p<0.05$) than the sediment from unheated solutions at the same pH, as shown in Fig. 4.1. Li-Chan (1983) stated that when temperature increases, electrostatic interactions and hydrogen bonds between whey protein molecules are weakened, while hydrophobic interactions are strengthened. Denaturation, aggregation and loss of solubility will increase if a high temperature is applied, since temperature and pH are interacting factors in the insolubility of β-lactoglobulin. After heating at 60°C, Li-Chan (1983) reported that no significant insolubilization was found in β-lactoglobulin, regardless of the pH; however, at 95°C a significant loss of solubility was found at all pH levels, but particularly at pH 8.

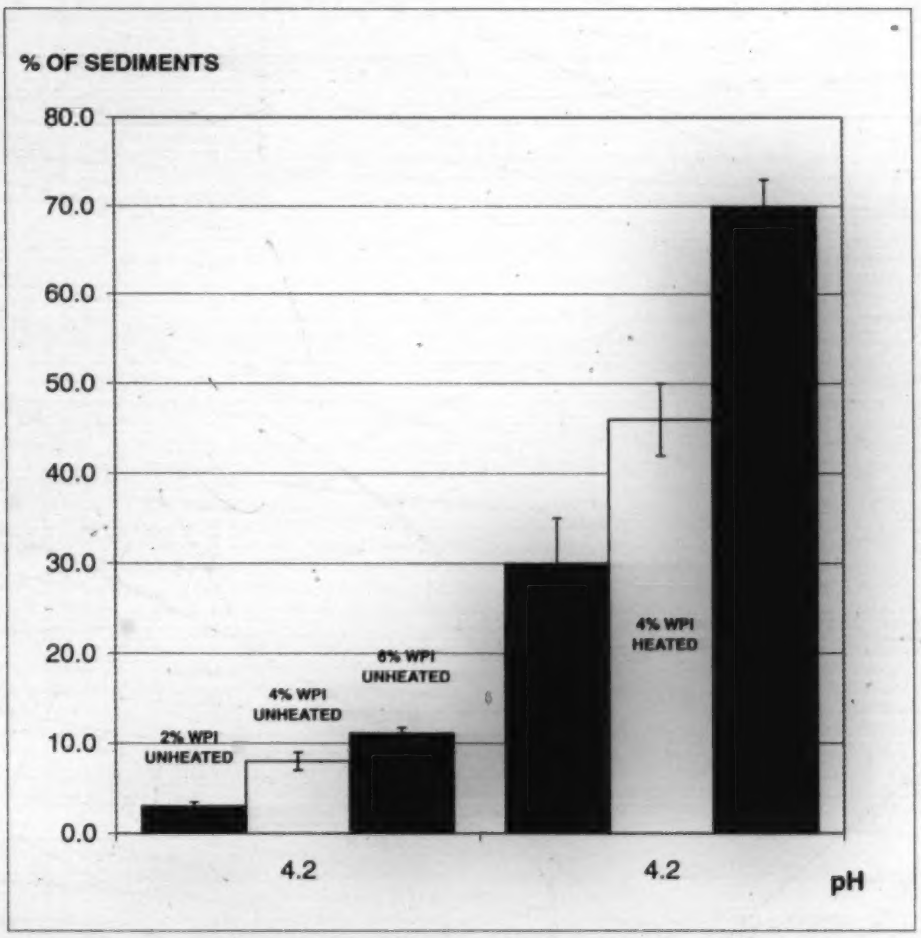

Figure 4.1: Visual sediments in 100 mL of unheated and heated aqueous solutions of WPI at pH 4.2

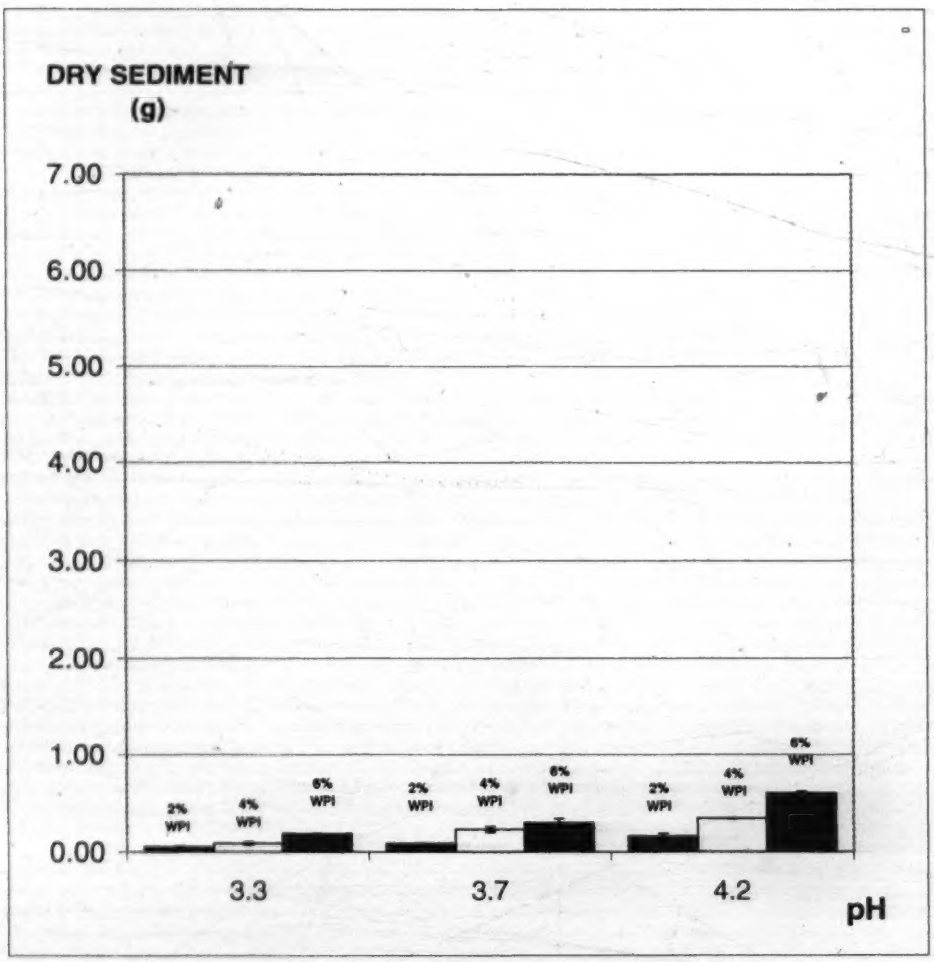

Figure 4.2: Dry sediments in 100 mL of unheated aqueous solutions of WPI at three pH

In our experiments, sedimentation was produced at all WPI concentrations and pH range in centrifuged heated solutions, as showed in Fig. 4.3. As in unheated systems, the amount of dry sediment was found to increase with the increased amount of WPI added at each pH. The amount of sediment obtained from heated solutions was significantly higher ($p<0.05$) than the sediments from unheated solutions at pH range 3.3-4.2. Jelen and Buchheim (1984) and Patocka et al. (1986) stated that acid whey heated at 92°C for 15 minutes below pH 3.5 showed a high resistance to coagulation and even lower turbidity than unheated whey samples, while heating in the pH 3.7-3.9 range produced great changes in the heat stability, even in diluted solutions having low ionic strength. Our results are consistent with those observations, since the volume of sediments obtained from heated solutions at pH 3.3 is small compared to those at pH 3.7 and 4.2.

4.2 Interactions in high methoxy (HM) pectin-WPI systems

4.2.1 Unheated HM pectin-WPI solutions

Serov et al. (1985) reported that there was no difference in the concentration of whey protein in a complex protein-pectin when using HM or low methoxy (LM) pectin. Since HM pectin is the most generally found form in tomato juice (Tiziani and Vodovotz, 2005), we decided to use only HM pectin in our experiments.

Liquid mixtures of WPI-pectin were prepared by adding 0.10, 0.20, or 0.30%HM pectin to 1%WPI solutions at pH 3.3, 3.7 and 4.2. Visual sediment was obtained at pH 3.3 and 3.7 at all concentrations of pectin, while no visual sediment was observed at pH 4.2. The volume of visual sediment increased with the increasing concentration of pectin added at

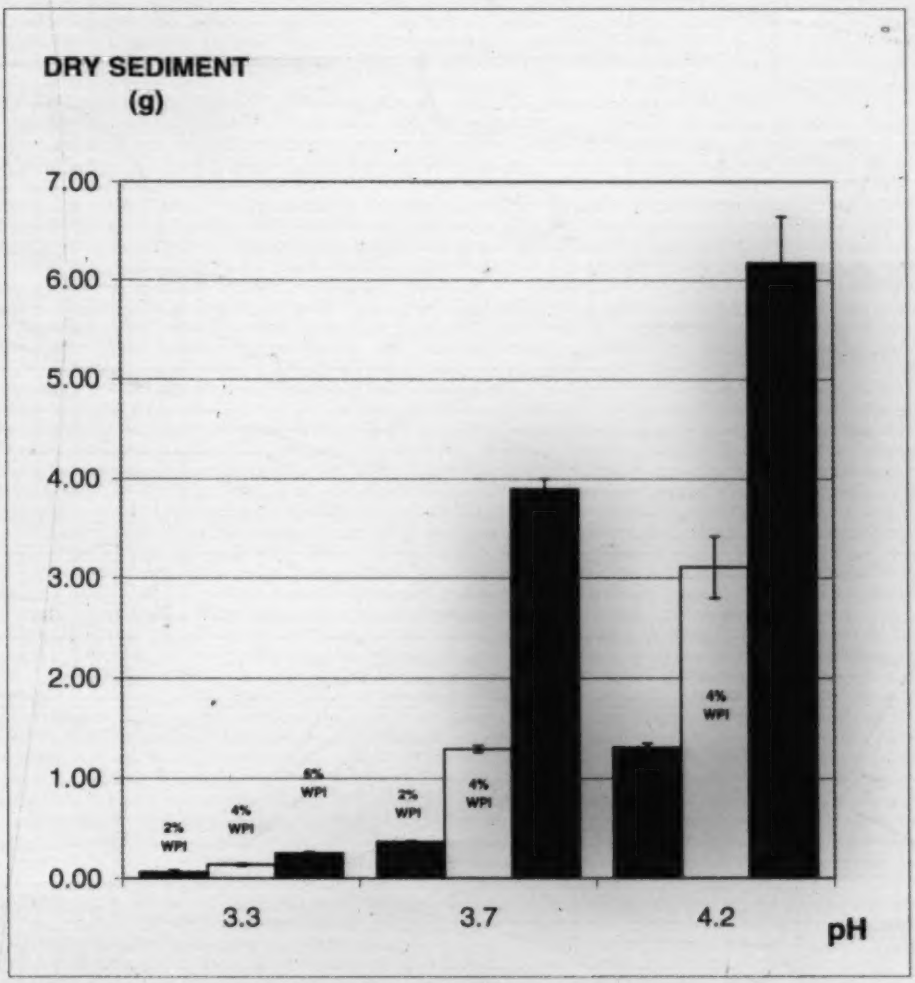

Figure 4.3: Dry sediments in 100 mL of heated aqueous solutions of WPI at three pH

the same pH. The highest volume resulted at pH 3.3, followed by pH 3.7, while at pH 4.2 no sediment was present, as shown in Table 4.1

Table 4.1 Percentage of visual sediments in 100 mL of solutions 1% WPI with different concentrations of pectin at three pH levels (the data are expressed as mean ± standard error of the mean, n=4).

UNHEATED SOLUTIONS			
Model system	pH		
	3.3	3.7	4.2
1% WPI + 0.10% pectin	5.0 ± 0.30	4.5 ± 0.15	0
1% WPI + 0.20% pectin	5.0 ± 0.50	4.9 ± 0.18	0
1% WPI + 0.30% pectin	7.0 ± 0.50	5.0 ± 0.53	0
HEATED SOLUTIONS			
1% WPI + 0.10% pectin	5.2 ± 0.30	4.6 ± 0.60	0
1% WPI + 0.20% pectin	10.1 ± 0.60	6.1 ± 0.40	0
1% WPI + 0.30% pectin	14.8 ± 0.75	0	0

While at pH 3.3 the supernatant was very clear, at pH 3.7 and 4.2 an increased turbidity was observed in the solutions. Further increase in the pectin concentration up to 0.50% caused no visual sedimentation at any pH level, but a noticeable turbidity in all solutions. After re-adjusting

the pH from 4.2 to 3.7 and 3.3, progressive sedimentation was observed in all solutions. This fact confirms that the interactions between whey proteins and pectin are mostly pH dependent.

The amount of dry sediment produced from 100 mL of centrifuged unheated solutions is shown in Fig. 4.4. Sediment was produced at all pH levels and in an increasing amount from solutions with an increased range (0.10% to 0.30%) of pectin. The lowest amount of sediment at a particular pH was obtained at pectin concentrations of 0.10%, and the highest at pectin concentrations of 0.30%. In the case of solutions with 0.10% and 0.20% pectin, the quantity of sediments increased noticeably from pH 3.3 to 3.7 and dropped drastically at pH 4.2. In contrast, the amount of sediment in solutions with 0.30% pectin decreased with increasing pH. The difference in the dry sediments at the three pH levels was significant ($p<0.05$) according to statistical analysis.

The sediments obtained from the 1% WPI control solution with 0% pectin clearly pointed out that the addition of pectin in the above mentioned range caused an obvious increment in the volume of sediments obtained at pH 3.3 and 3.7, nevertheless, the amount of sediment produced at pH 4.2 is similar to the sediment from the control sample. This result was presumably caused by a lower interaction between oppositely charged proteins and pectin as the pH rose, decreasing the positive electric charge of the protein and increasing the repulsive forces between the molecules, thus reducing the development of the protein- pectin complex, and/or due to a progressive increase in the viscosity of the system, leading to lower sedimentation of the particles. Takada and Nelson (1983) found radical changes in the viscosity depending on the pH of a whey protein-pectin solution. In the pH range 3.5 – 5.0 they found the maximum viscosity at pH 4.2. Previous viscosity

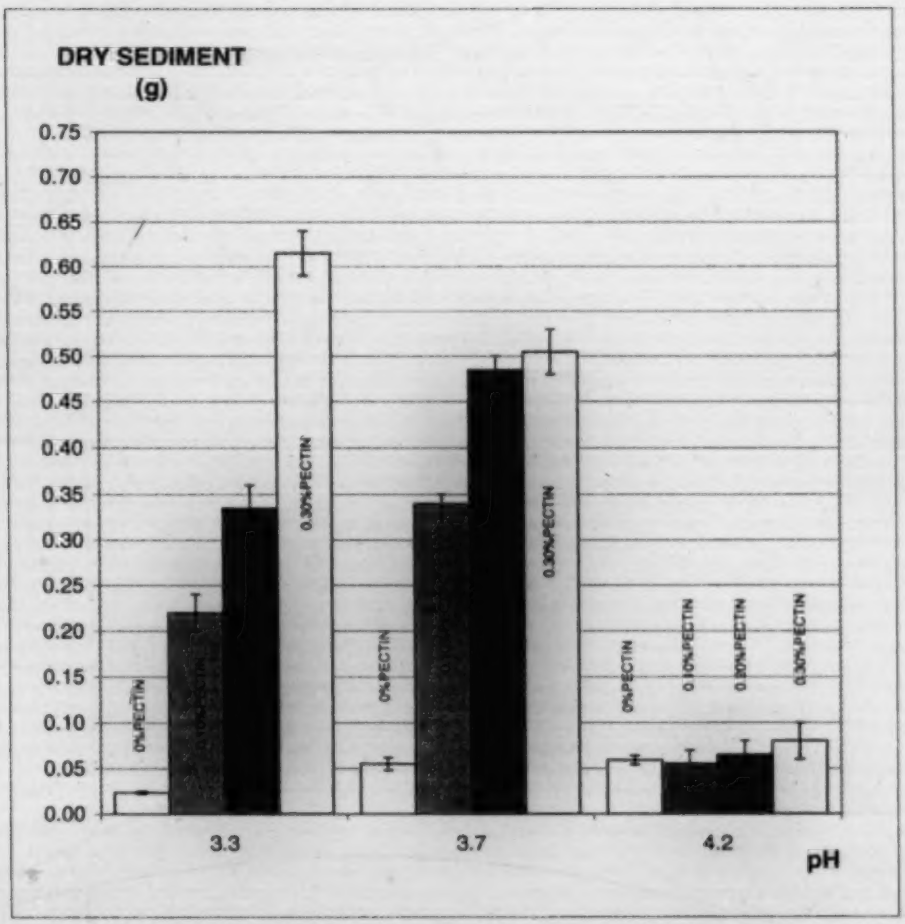

Figure 4.4: Dry sediments in 100 mL of unheated solutions of 1% WPI with 0.10%, 0.20%, or 0.30% pectin

determinations of pectin or whey protein in separate solutions in the same pH range did not show any change. Dickinson (1998) stated that the complexation between protein and polysaccharide molecules is produced by electrostatic interactions. The linkage generated between proteins and carboxylated polysaccharides, such as pectin, is much stronger if it is produced at pH values lower than the isoeletric point of the protein.

Analysis of the pectin content in the sediments showed that the amount of pectin increased with the increasing concentration of pectin at a particular pH. The lowest relative pectin content was found in the sediment of solutions with 0.10% of pectin, followed by 0.20% and 0.30%. The relative pectin content was found to be slightly higher in solutions at pH 3.3 than at pH 3.7 at the same pectin concentration as depicted in Fig. 4.5, even if the difference was found statistically not significant ($p>0.05$). Since the purpose of these experiments was to study the interactions between whey proteins and pectin, the pectin content of sediments was determined only in solutions at pH 3.3 and 3.7 and not in solutions at pH 4.2, owing to sedimentation of whey proteins observed in unheated and heated whey-water solutions at pH 4.2 even in the absence of pectin.

These results confirm a clear interaction between whey proteins and pectin which is pH dependent. The addition of pectin into the WPI solutions indeed produced an insoluble complex between whey proteins and pectin.

Serov et al (1985) stated that an insoluble complex was formed from macromolecular components with opposite charges as a result of the interaction between whey proteins and apple pectin. Imeson et al. (1977) reported that the principal forces responsible for the interactions between bovine serum albumin (BSA) and anionic polysaccharides, such as pectate, alginate, and carboxymethyl cellulose (CMC), are of electrostatic nature and pH dependent, increasing with reducing pH as a

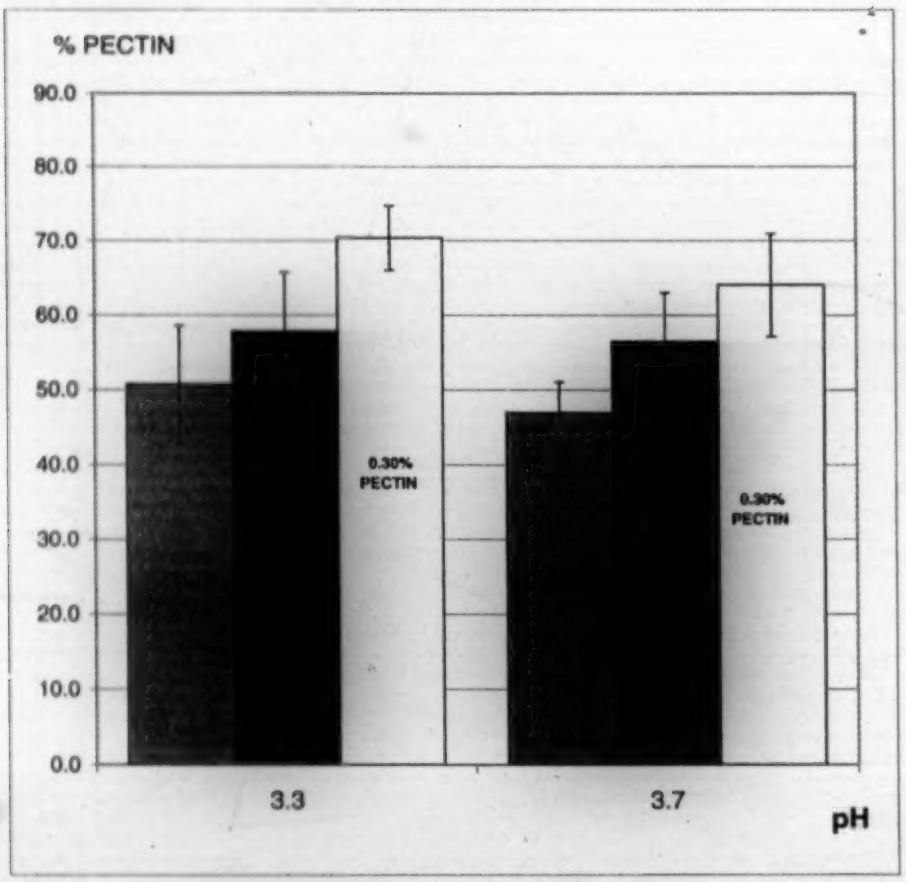

Figure 4.5: Relative pectin content in the sediments of unheated solutions of 1% WPI with 0.10%, 0.20%, or 0.30% pectin

result of increasing positive charge on the protein. Imeson et al. (1977) and Girard et al. (2002) proposed that these electrostatic interactions are generated between the negatively-charged carboxylate groups of the polysaccharide and the positively-charged amino, guanidinium and imidazole groups of the protein, and the distribution, number of these sites, and the net charge of the protein, are related to the strength of the interactions. On the other hand, Dickinson (1998) stated that, owing to the lower proportion of carboxylic groups, HM pectin produces weaker electrostatic interactions with proteins than LM pectin. The interactions between whey proteins and pectin will increase as the net charge of the proteins becomes more negative as a result of the reduction in the pH. However, since these interactions are also related to the carboxylic groups in the pectin, the reduction in the pH should be limited to the point where the dissociation of the carboxylic groups is drastically reduced, avoiding the complexation (Ganzevles et al., 2005). Because variable percentages of pectin were found in the sediment of our whey protein-pectin solutions, it may be thought that the other component in the sediment is whey protein. Hansen et al. (1970) recovered 60% of protein and 30% of CMC from the dry sediment of a whey protein-CMC solution at pH 3.2. Devkota (1991) analyzed the protein and pectin contents of sediments of 1% whey protein concentrate (WPC) solutions with added pectin in a 0.1-0.3% range at pH 3.4 and 3.7. The pectin content in the sediment increased with the addition of pectin, while the protein content decreased. The concentration of pectin in the sediments was higher at pH 3.4 than at pH 3.7, whereas a proportionally lower percentage of protein was found at pH 3.4 than at pH 3.7.

From our experiments, we observed that the sediment in the system WPI-pectin was insoluble in water, probably due to the interactions between proteins and pectin leading to the development of

insoluble complexes. The visual sedimentation in the whey protein-pectin system may occur owing to the formation of an insoluble protein-pectin complex with a molecular weight greater than the single molecular weight of the protein or pectin molecules. Girard et al (2002) observed that after the ultrafiltration by centrifugation of a β-lactoglobulin-pectin solution, a complex formed by β-lactoglobulin and HM pectin was retained, while the non-complexed proteins passed through the membrane. Beaulieu et al. (2005) found that mixing whey protein and pectin increased the average molecular weight of the complex; they also observed that the effect was the same regardless the demethylation degree of the pectin. Imeson et al. (1977) reported the formation of a complex after adding bovine serum albumin into pectate solutions which had a higher molecular weight than the molecular weight of the native protein.

4.2.2 Heated HM pectin-WPI solutions

After heating 0.10% to 0.30% HM pectin and 1%WPI solutions at pH 3.3, 3.7 and 4.2, the amount of visual sediment at pH 3.3 increased at all pectin concentrations, especially at 0.20% and 0.30%, compared to the sediment from unheated solutions (Table 4.1), the overall variation in the visual sediments between unheated and heated solutions at each pH was significantly ($p<0.05$) different. However, at pH 3.7 and concentrations of pectin of 0.10% and 0.20%, the quantity of sediment from heated solutions was a little higher than the sediments in unheated ones at the same pH, while at 0.30% pectin concentration, there was no visible sedimentation in heated solutions. At pH 4.2, as in unheated solutions, there was an absence of any visual sediment at all pectin concentrations. The visual sediment produced in heated solutions had the same tendency to decrease when the pH increased (the highest amount

at pH 3.3 followed by pH 3.7 and absence at pH 4.2) as noted in unheated mixtures, as shown in Table 4.1.

The dry sediment in heated solutions increased with the addition of pectin (0.10, 0.20, or 0.30%) at pH 3.3 and 3.7 as represented in Fig. 4.6. At pH 4.2, on the contrary, the sediments decreased when the concentration of pectin increased. The fact that at this pH the highest amount of sediment (0.61± 0.030 g) was obtained in the solution with 0% pectin, followed in decreasing order by the solutions with 0.10, 0.20 or 0.30% of pectin, suggests that the sediment is mainly produced by the precipitation of the whey proteins at pH 4.2, and the increased concentration of pectin in heated solutions at this pH had the effect of protecting the heated whey protein from precipitating. The amount of dry sediment of heated solutions showed to be significantly higher ($p<0.05$) than the dry sediment of unheated solutions at the three pH levels studied.

According to our results, the lowest amount of dry sediment in unheated and heated WPI-pectin solutions was produced at pH 4.2. It must be pointed out, however, that the kind of sediment observed from unheated and heated systems was different. Even if no changes in color were noted, the unheated sediments looked weak and soft, whereas curd-type sediments were obtained from heated mixtures.

Einhorn-Stoll et al. (2004) described the formation of brown color, probably due to a Maillard-type reaction, after mixing whey proteins and HM pectin and heating the mixture at 60-65°C. They reported that HM pectin produced significantly more brown color conjugates than LM pectin did. Imeson et al. (1977) stated that after the thermal denaturation of the protein in a protein-anionic polysaccharide solution, the availability of amino, guanidine, and imidazole groups increases, resulting in the maximization of the interaction with the carboxylated polysaccharides.

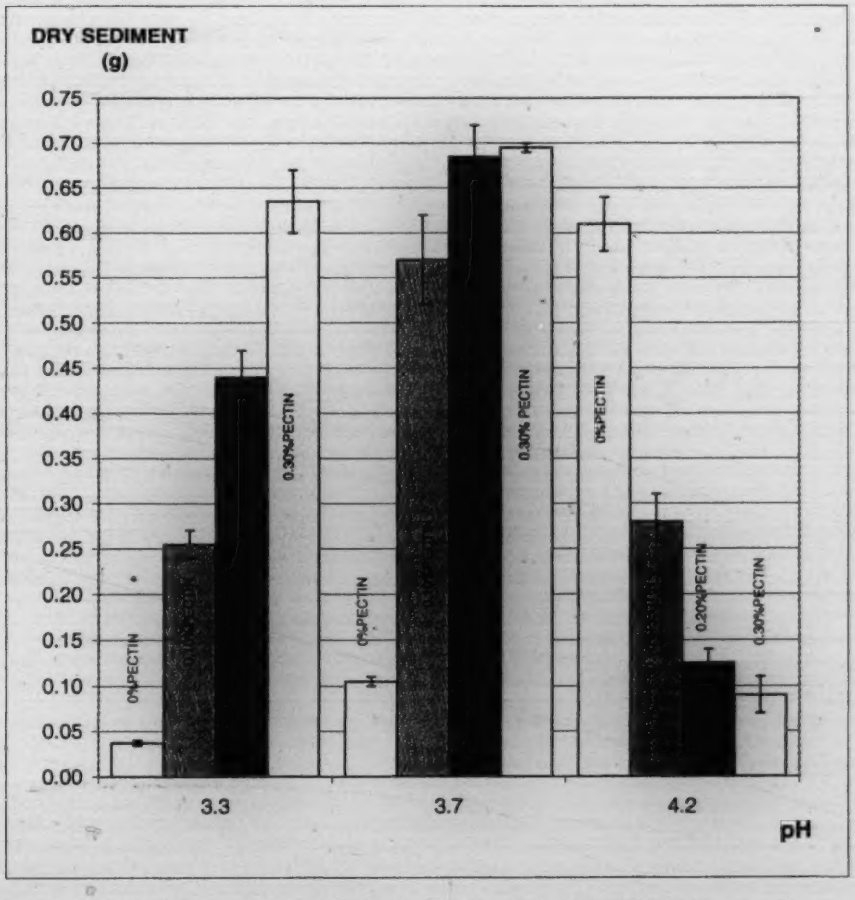

Figure 4.6: Dry sediments in 100 mL of heated solutions of 1% WPI with 0.10%, 0.20%, or 0.30% pectin

This effect, in addition to the adjustments in the configuration produced by the flexibility of the random coil of the denatured protein molecules, leads to the formation of more stable complexes compared to those produced with unheated proteins. Galston and Kaur (1962) stated that the heat coagulation of proteins at pH values lower than 5, was facilitated by pectin, while Takada and Nelson (1983) reported on the contrary, that the heating of a system containing whey proteins and pectin produced a more stabilized and irreversible complex between the denatured protein and pectin.

The relative pectin content of sediments following the heating of solutions was found to increase with an increased concentration of pectin at both pH levels. As in unheated solutions, the lowest pectin content was obtained from blends containing 0.10% of pectin, and the highest from blends containing 0.30% of pectin. The pectin content in the sediments of solutions at pH 3.3 was higher than the pectin content in the sediments at pH 3.7, as depicted in Fig 4.7. The pectin content in the sediments at pH 4.2 was not determined due to the very small amount of sediment obtained, and because of these sediments were produced at this pH even in heated solutions without pectin. However, the relative pectin content in the sediment of heated mixtures was lower than the pectin content in the sediment of unheated mixtures. Further statistical analysis showed a significant decrease ($p<0.05$) in the pectin content of sediments from heated solutions compared to the pectin in the sediments of unheated ones. This effect was probably caused by the denaturation of the proteins resulting in an increased proportion of whey proteins in the complex as the pH rose.

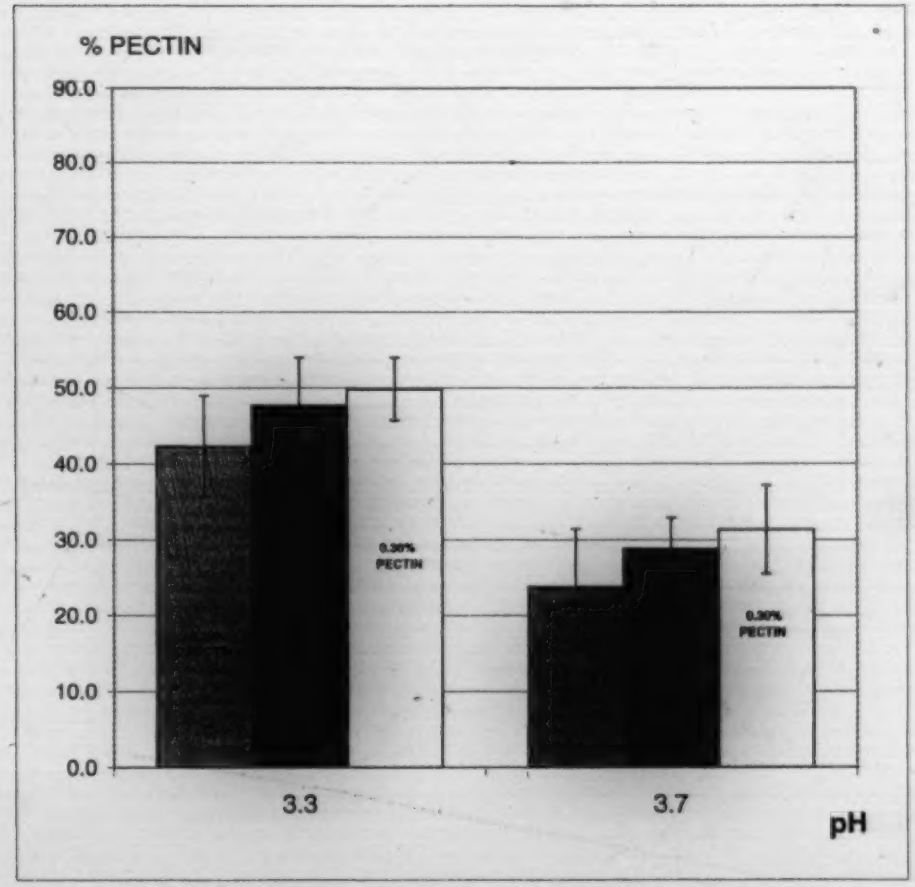

Figure 4.7: Relative pectin content in the sediments of heated solutions of 1% WPI with 0.10%, 0.20%, or 0.30% pectin

4.3 Whey protein-pectin interactions in tomato juice-whey protein mixtures

Fresh tomato juice was prepared in the laboratory according to the "cold brake" process (Gould, 1983) and also tried, but was found to give similar results as canned tomato juice, so canned tomato juice was used and is generically referred as "tomato juice".

4.3.1 Unheated tomato juice-WPI mixtures

Mixtures containing 2%, 4%, or 6% WPI were prepared at 20°C by adding the WPI into tomato juice and adjusting the pH to 3.3, 3.7, and 4.2. No visible sediments were obtained at any pH or WPI concentrations, although a slight change in the color of the mixtures was noted. As WPI was added to the mixtures, the deep red color of the tomato juice turned proportionately lighter red (or pink).

The sediments obtained by centrifugation were subsequently dried. There was a progressive increase in the amount of dry sediments produced at each pH as the concentration of WPI increased. The highest amount of sediment at the three WPI concentrations (2%, 4%, or 6%) was obtained at pH 3.3, followed by pH 3.7 and 4.2 as represented in Fig. 4.8. The amount of dry sediment was significantly different ($p<0.05$) among the three pH levels. This result shows a trend similar to that obtained from previous experiments with unheated 1%WPI-0.30% pectin solutions, although the system WPI-tomato juice is quite different, taking into account that tomato juice is an intricate mixture of pulp and serum portions containing pectin, cellulose, fibers and other carbohydrate polymers, besides protein and minerals (Gould, 1983; Xu et al., 1986; Leroux et al., 2003).

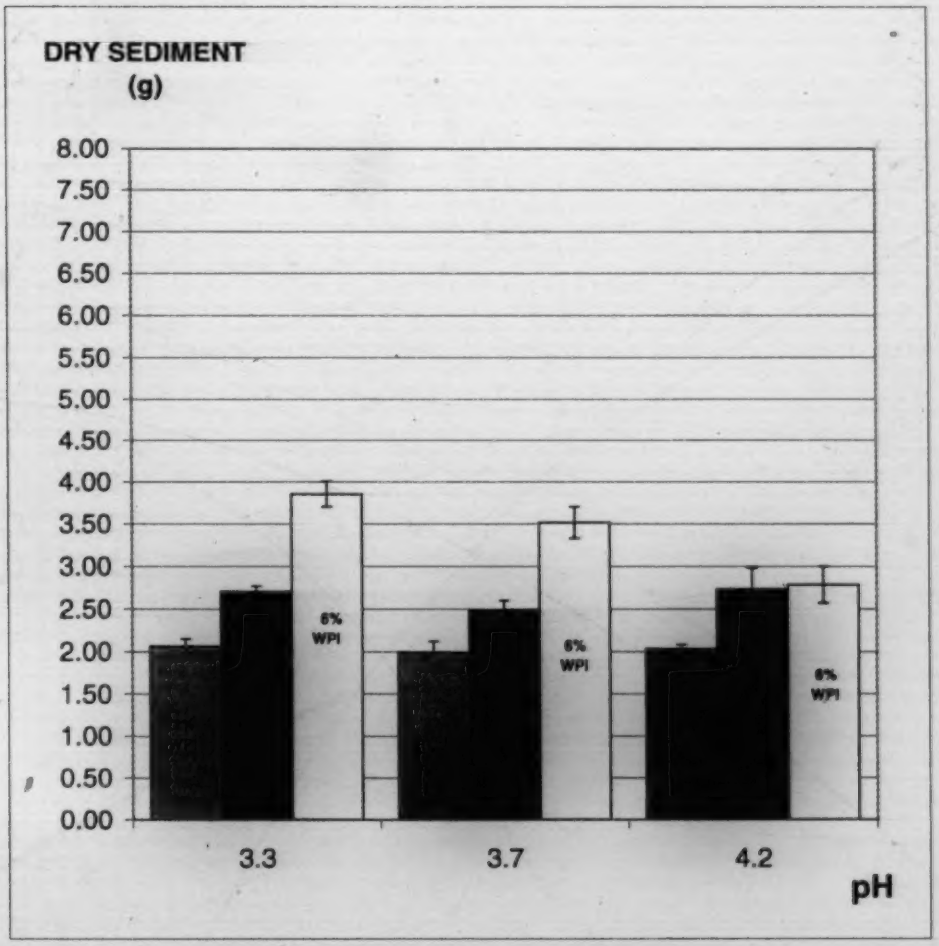

Figure 4.8: Dry sediments in 100 mL of unheated mixtures of 2%, 4%, or 6% WPI with tomato juice

The analysis of the protein content in the dry sediments indicated an increasing percentage of protein with increased concentration of WPI at pH 3.3 and 3.7. As mentioned before, the protein content at pH 4.2 was not determined since whey proteins precipitated at this pH even when pectin was not present. Fig. 4.9 depicts the relative protein content in the different sediments with 2, 4, or 6% concentrations of WPI at each pH. A considerably higher amount of protein ($p<0.05$) was found in sediments at pH 3.7 than at pH 3.3. On the other hand, measurements of the relative pectin content of dry sediments, found higher pectin concentration in mixtures at pH 3.3 and lower in mixtures at pH 3.7, in an inverse and complementary trend to that observed in the protein content. The difference in the pectin content in the sediment showed to be significant ($p<0.05$) among the different WPI concentrations but not between the two pH levels (Fig. 4.10). The generally low level of relative protein and pectin contents (Fig. 4.9 and 4.10) is obviously due to other insoluble components of the complex mixture sedimenting upon centrifugation.

Single-point measurements of the viscosity of 2, 4, or 6% WPI-tomato mixtures were carried out at 20°C using a rotary viscometer, to study the effect caused by variable concentrations of undenatured whey proteins in the tomato juice on the apparent viscosity of a commercial juice. The viscosity in the unheated mixtures increased with the increasing amount of WPI added at pH 3.3 and 3.7, while it decreased at pH 4.2 within the studied range (2%-6%) as showed in Fig. 4.11. Sharma et al. (1996) reported that the viscosity of tomato pulp was directly related to the total solids content, among other factors such as pectin and water-insoluble solids concentration. The overall trend of our results is in agreement with this statement, since the highest amount of sediments coincided with the highest viscosity in solutions at pH 3.3 and 3.7, while the lowest amount of sediment and the lowest viscosity was found at

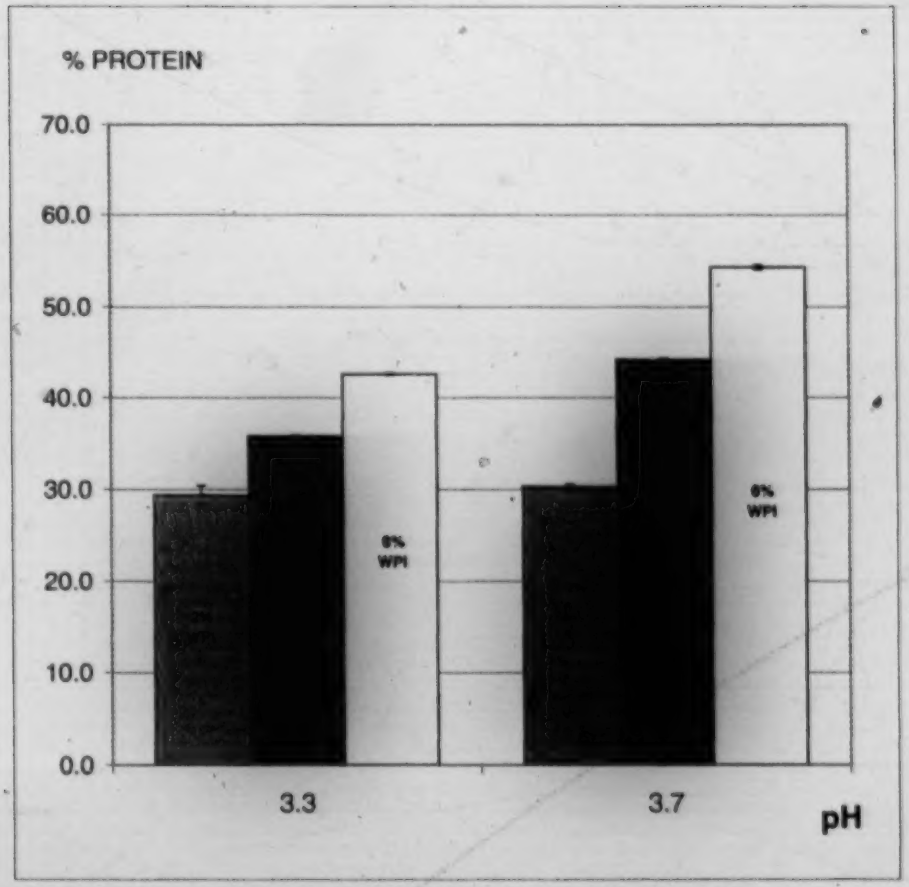

Figure 4.9: Relative protein content in the sediments of unheated mixtures of 2%, 4%, or 6% WPI with tomato juice

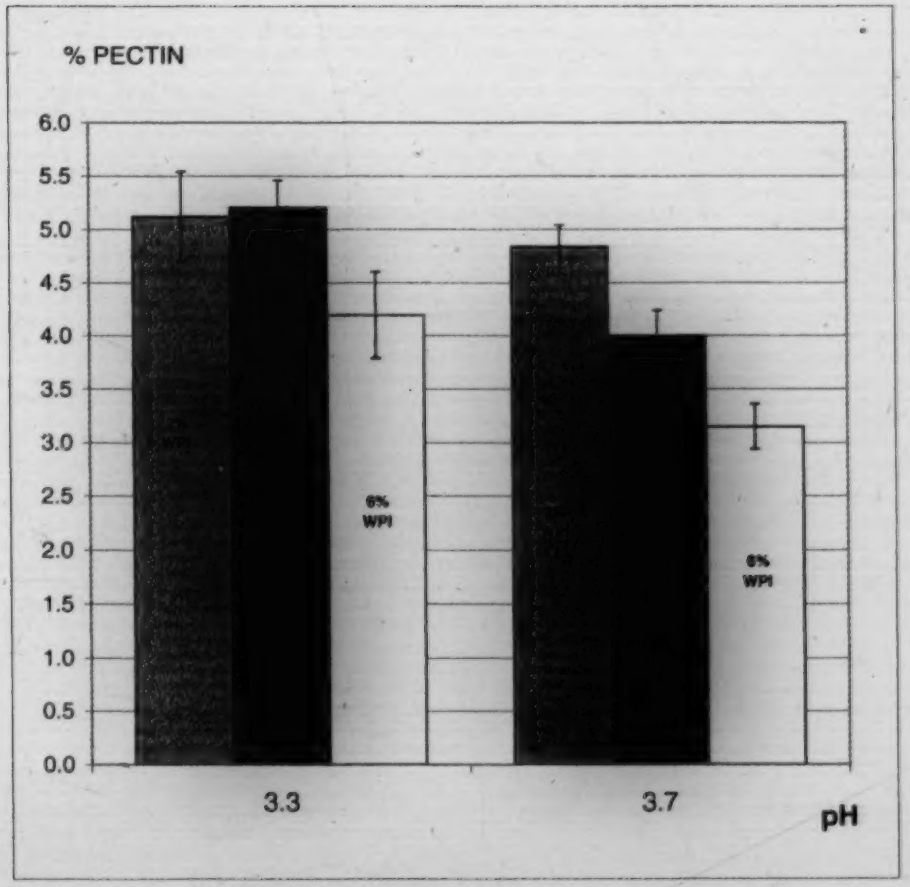

Figure 4.10: Relative pectin content in the sediments of unheated mixtures of 2%, 4%, or 6% WPI with tomato juice

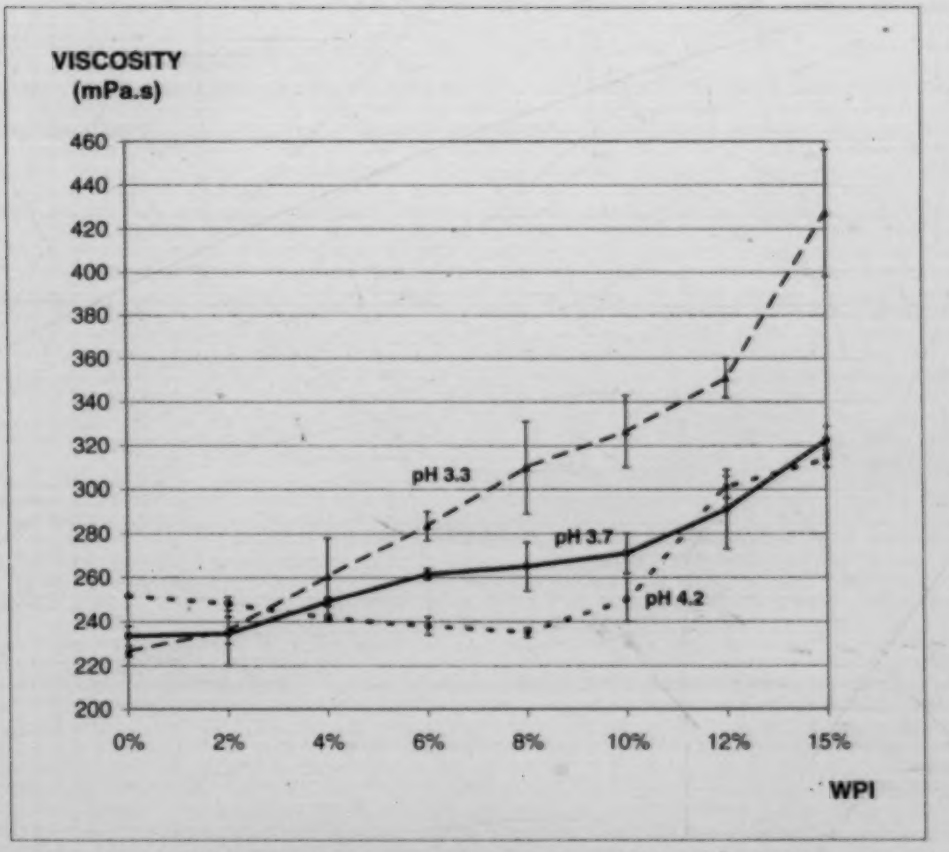

Figure 4.11: Viscosity of unheated mixtures of 0%-15% WPI and tomato juice at three pH levels

pH 4.2. The viscosities of the 2%-6% WPI-tomato juice mixtures were different at the limits of the pH range (3.3 and 4.2) studied.

Taking into consideration the variation in the apparent viscosity caused by the amount of WPI at different pH, it was decided to prepare additional mixtures containing 8, 10, 12, or 15% WPI in tomato juice at the three pH levels (3.3, 3.7, and 4.2) to study the effect of higher concentrations of whey proteins in the viscosity of the solutions. It was found that the viscosity in the mixtures with WPI concentration ranging from 8% to 15% at pH 3.3 and 3.7 followed the same tendency as previously observed; i.e., the viscosity increased with the increasing amount of WPI in the solutions. In contrast, the viscosity of mixtures at pH 4.2 presented a continuous decrease up to a concentration of 8% WPI. From concentrations higher than 8% and up to 15% of WPI, the viscosity values rose with the increasing amount of WPI in the mixtures, following the same tendency observed at pH 3.3 and 3.7 (Fig.4.11).

Patocka et al. (2006) found that the addition of WPI up to 8% into commercial yoghurt drink lowered the viscosity of the mixture, probably due to the fact that the highly water soluble WPI disrupts the spatial configuration of H_2O molecules, thus producing more fluidity. Considering that the pH of different types of yoghurt and kefir is in the vicinity of pH 4.5 (Bylun, 1995a), the viscosity trend of our unheated WPI-tomato mixtures at pH 4.2 is in accordance with these results.

From our observations we assume that there is an obvious relation between the viscosity of the mixtures, the protein concentration, and the pH. It may be thought that a kind of network structure was probably formed between the undenatured proteins and the tomato juice pectin, and changes in the ratio protein/pectin in the system would have a direct effect in the strength or weakness of this network at a specific pH. An explanation for the reduction in the viscosity after increased incorporation

of WPI into the tomato juice at pH 4.2 could be that of Tolstoguzov (1995), who stated that undenatured whey proteins participate as inactive filler if they fail to form a consistent network with the pectin of the tomato juice, causing a decrease of the mechanical properties of the mixture: i.e, a weakness of the network between the two polymers which at the time causes a drop in the viscosity of the system. This may be the case in our WPI-tomato mixtures at protein concentrations up to 8%, while, on the contrary, it can be suggested that a progressive strength in the cohesivity of the network occurred at pH 3.3 and 3.7, increasing the viscosity as the concentration of proteins rose.

4.3.2 Heated tomato juice-WPI mixtures

The heating of the mixtures of WPI and tomato juice produced no visual sediments; instead, two effects were observed. First, the color of the mixtures turned a lighter red, probably due to the partial destruction of the lycopene and other carotenoids (Gould, 1983; Riso et al., 2006). Second, the particles in suspension seemed to turn into a coagulum form distributed uniformly in the solution. The dry sediments in the heated mixtures at 6% WPI (but not at other WPI concentrations) presented the same trend as in unheated mixtures: i.e., the highest amount of sediment occurred at pH 3.3, followed by pH 3.7 and 4.2, as shown in Fig. 4.12. The amounts of dry sediments from heated mixtures were significantly higher ($p<0.05$) at all pH levels and WPI concentrations than the sediments of unheated mixtures. As in unheated samples, the relative protein content in the dry sediments of heated mixtures increased as the concentration of WPI increased. As depicted in Fig. 4.13, the level of protein was markedly higher ($p<0.05$) in sediments at pH 3.7 than at pH 3.3.

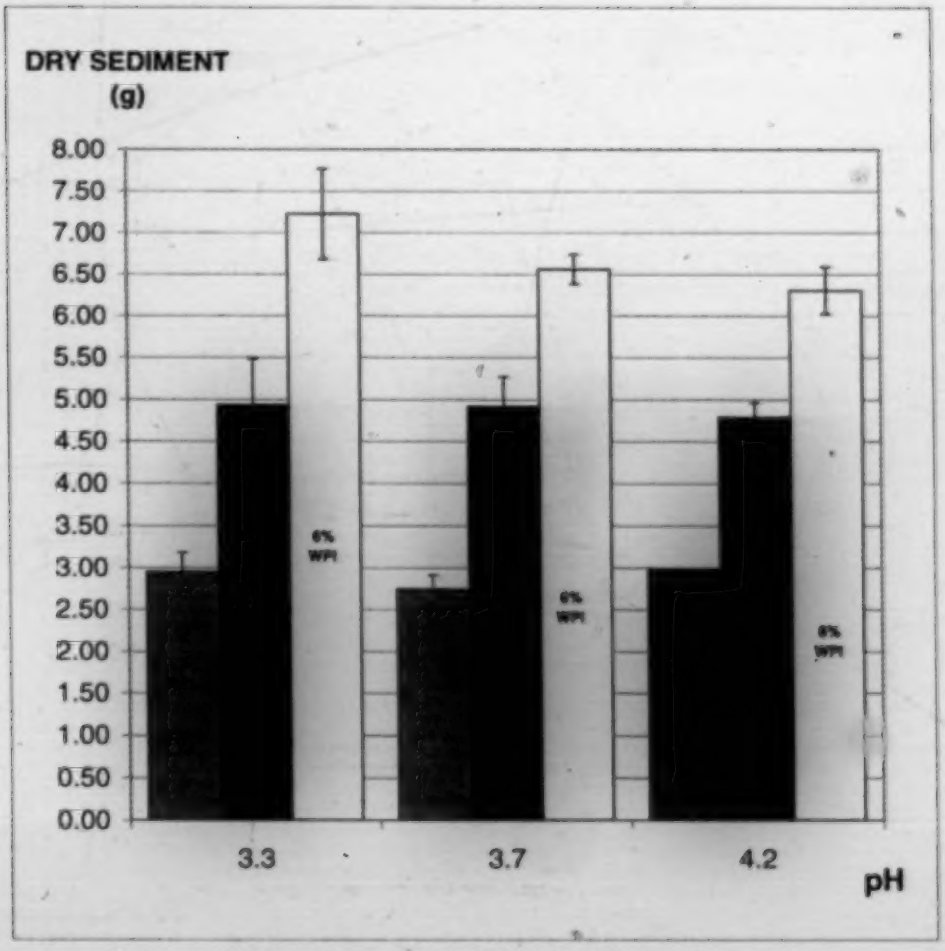

Figure 4.12: Dry sediments in 100 mL of heated mixtures of 2%, 4%, or 6% WPI with tomato juice

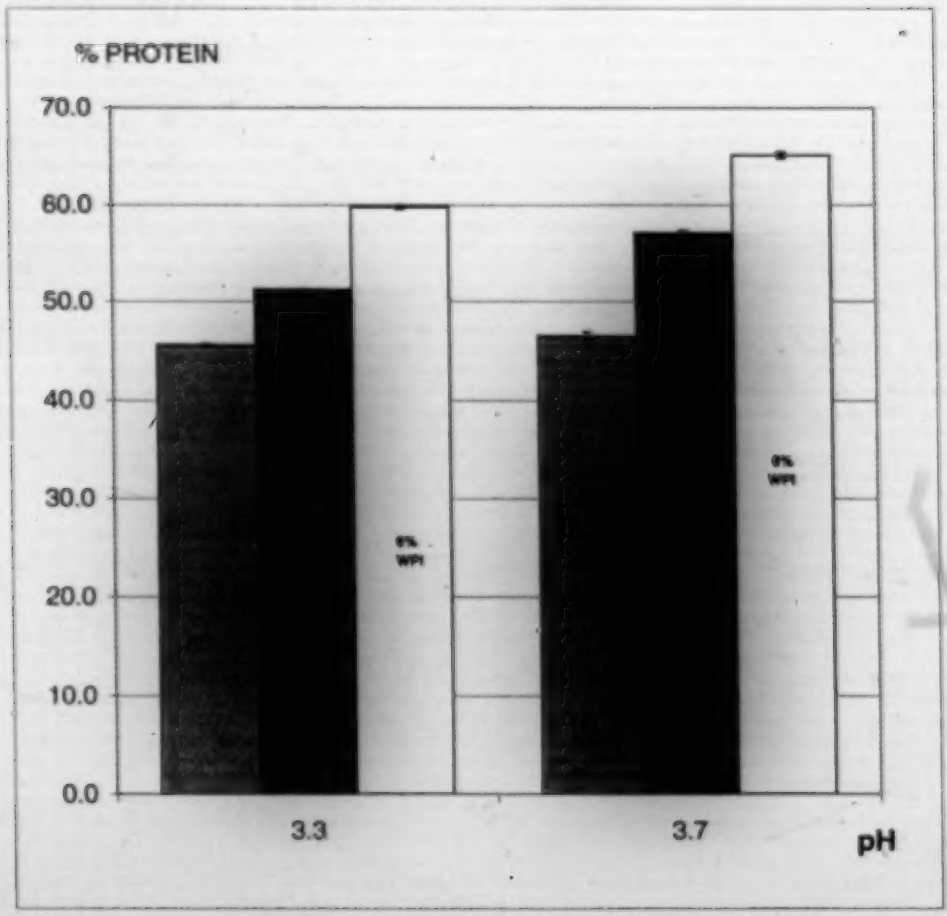

Figure 4.13: Relative protein content in the sediments of heated mixtures of 2%, 4%, or 6% WPI with tomato juice

The protein content in the sediment in heated solutions was drastically higher ($p<0.05$) than in unheated solutions. On the other hand, the relative pectin content in the dry sediments of heated WPI-tomato mixtures was higher at pH 3.3 than at pH 3.7, as Fig. 4.14 depicts. The pectin content of the sediments of heated mixtures tended to be lower than the pectin content of the sediments of unheated mixtures, even if the variation tested not to be significant ($p>0.05$).

The effect of heating on the viscosity of these mixtures is represented in Fig. 4.15. In heated solutions at pH 3.3, 3.7 and 4.2, there was a constant increase in the viscosity at each pH when the amount of WPI added to the mixture increased. The lowest viscosity was found at pH 3.3 and the highest at pH 4.2, and the viscosity of the heated mixtures was clearly higher than the viscosity of unheated mixtures at all WPI concentrations and pH levels. A similar trend was observed by Tiziani and Vodovotz (2005) who found a significant increase in the viscosity of a heated mixture (100°C for 10 min) of tomato juice and 1% soy protein isolate at pH 4.19. These results contrast with the amount of dry sediment found in heated mixtures, where the highest amount of dry sediment was observed at pH 3.3 (the lowest viscosity), while the lowest amount was produced at pH 4.2 (the highest viscosity).

On the other hand, we found that the viscosity of unheated tomato juice is higher than of the heated one. Fig. 4.16 depicts the significant difference ($p<0.05$) in the profile of the viscosity between heated and unheated tomato juice at pH 4.2 (the natural pH of the tomato juice).

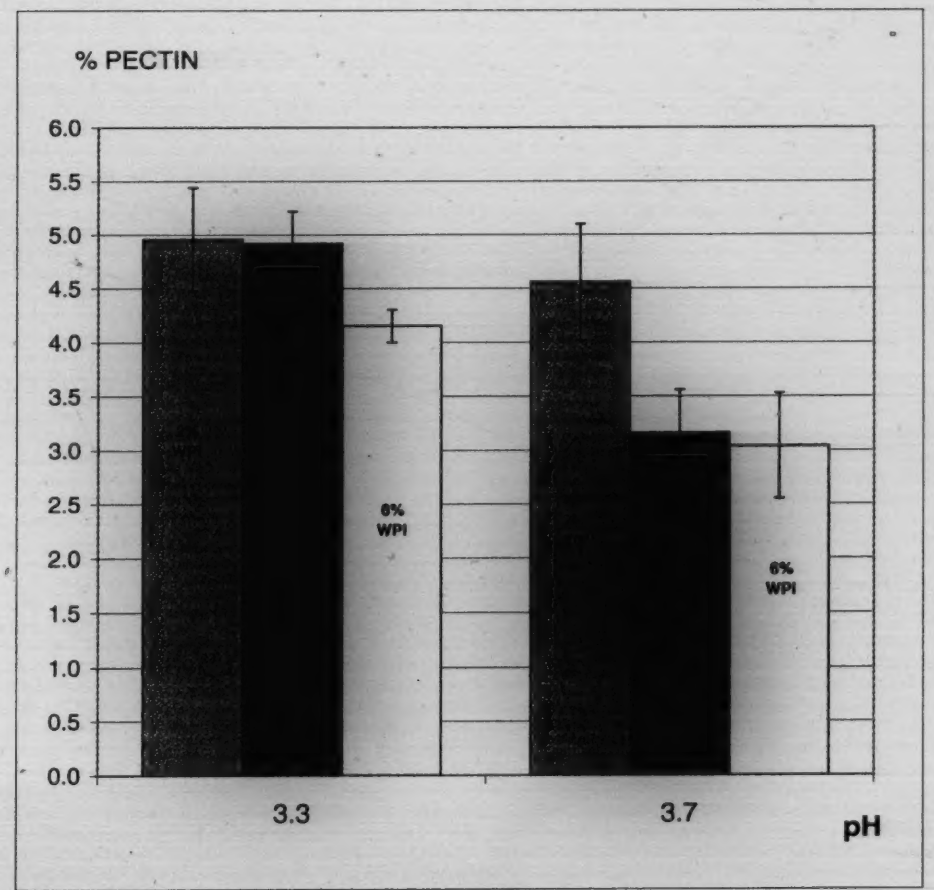

Figure 4.14: Relative pectin content in the sediments of heated mixtures of 2%, 4%, or 6% WPI with tomato juice

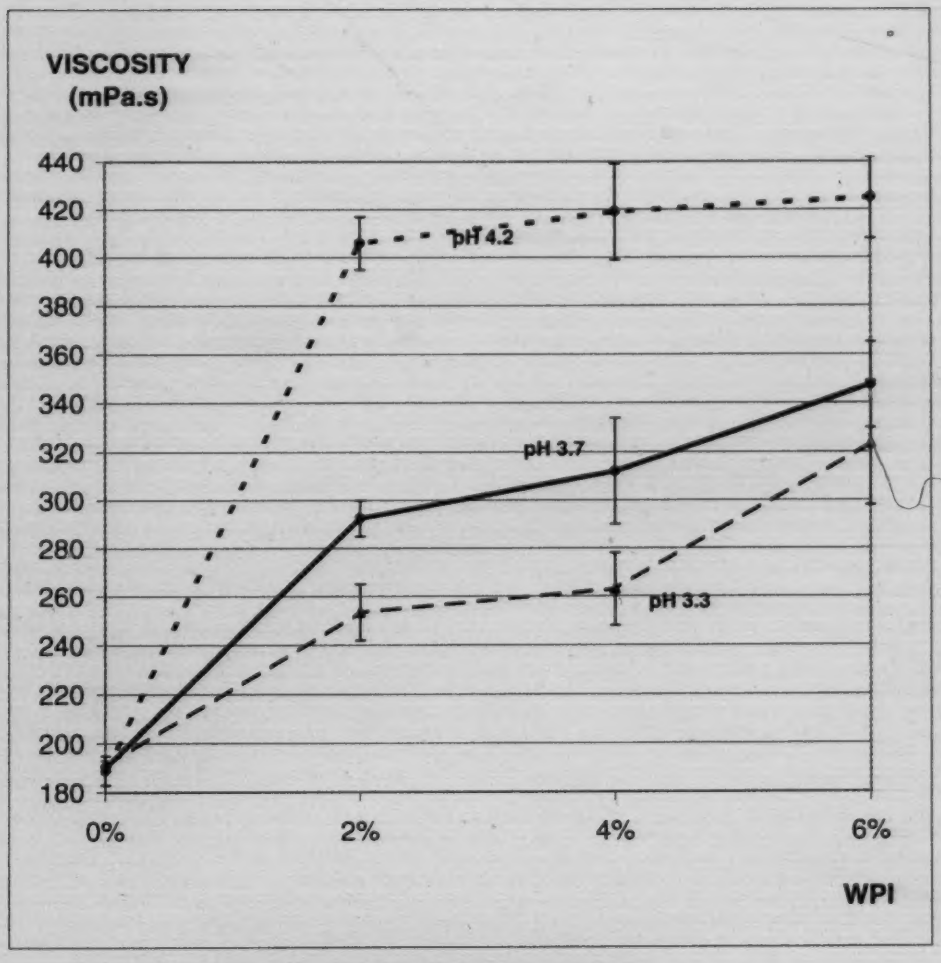

Figure 4.15: Viscosity of heated mixtures of WPI and tomato juice at three pH levels

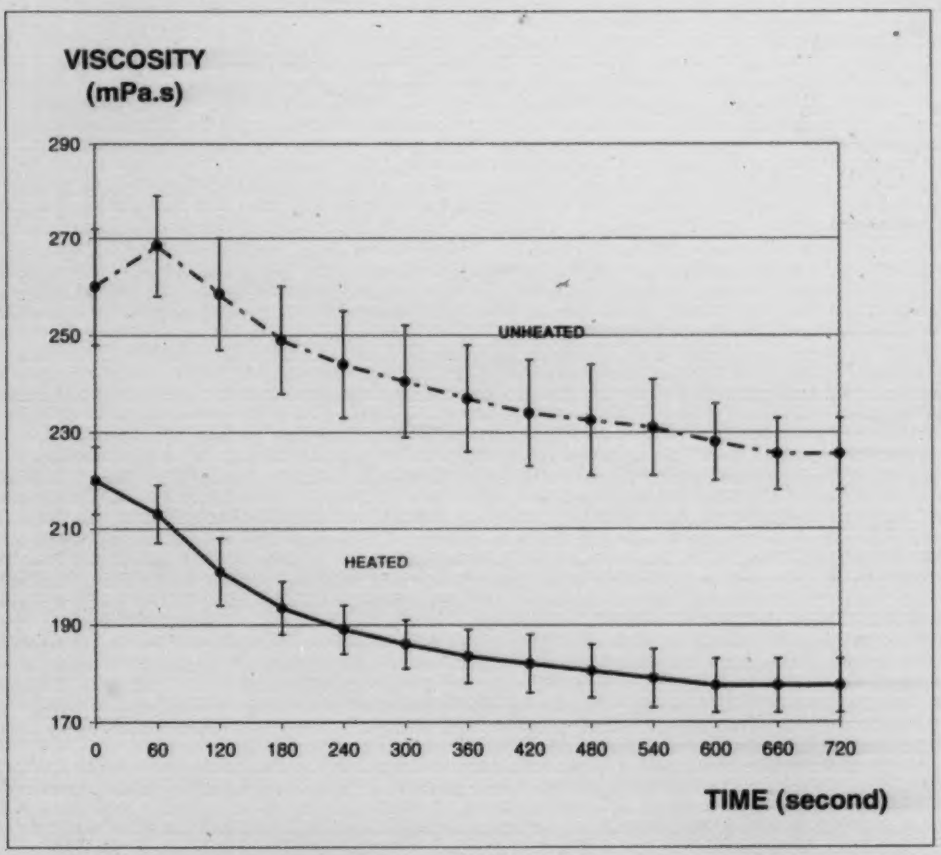

Figure 4.16: Viscosity profile of unheated and heated tomato juice at pH 4.2

Chou and Kokini (1987) reported a reduction in the viscosity of solutions containing pectin from hot break tomato paste when the pH decreased from 4.6 to 2.6. They stated that at pH 2.6 some deesterification of the pectin chain may occur due to the acid condition, leading to a decrease in the viscosity. On the other hand Axelos and Branger (1993) reported a decreasing in the viscosity of HM pectin solutions as the temperature increased. They also stated that HM pectin undergoes a greater decrease in the viscosity than LM pectin for a given temperature. From these outcomes it is possible to suggest that the reduction in the viscosity of our tomato juice samples following heating at pH range 3.3-4.2, is probably caused by the breakdown of the pectin chain, causing a decrease in the interactions between the pectin and the solvent, leading to the drop in the viscosity. An additional effect caused probably by the deesterification of pectin molecules in acidic conditions would explain the decrease in the apparent viscosity of the unheated juice when the pH decreased from pH 4.2 to 3.3. However, when WPI is added to the tomato juice and the mixture is heated, probably a stronger cohesive network is formed among proteins, pectin, and other polymers, which is strengthened by the increased number of protein groups available to interact.

4.3.3 Enzymatic treatment of pectin in tomato juice

The blend of WPI and tomato juice produces complexation of whey proteins and tomato pectin, which are components in the sediment of these mixtures. The enzymatic treatment of pectin was studied as a tool to reduce the pectin effects on the sediment formation. The action of polygalacturonase enzyme in the hydrolysis of pectin was measured by analysis of the pectin content in the sediments of treated tomato juice

alone and mixed with WPI at pH 4.2, the natural pH of tomato juice. Alternatively, the effect of this enzymatic treatment was also studied by measuring the changes on the apparent viscosity of the tomato juice and WPI-tomato juice mixtures.

According to Brummell and Harpster (2001) and Jayani et al., (2005) polygalacturonase is the main enzyme participating in the depolymerization of pectin polysaccharides. Its pectolytic action involves the cleavage of the polygalacturonic acid chain of the pectin molecules. To study the time of action of polygalacturonase on tomato juice, the enzyme in concentrations of 0.05% or 0.50% was added to samples of tomato juice at 40°C. Consecutive viscosity measurements were carried out every hour for the next 12 hours.

Fig 4.17 illustrates the change in the viscosity of treated tomato juice and untreated control sample (without enzyme). The viscosity of the control juice stabilized within the first 10 minutes, showing a constant value after that time. On the contrary, the viscosity of the two samples treated with polygalacturonase decreased with time, reaching the lowest value after 12 hours. The action of polygalacturonase was clearly higher during the first 5 hours in both treated samples, showing a continuous reduction of the viscosity. Between the hours 6 and 10, the viscosity was reduced more gradually, evidencing a tendency to the stabilization after the hour 10, probably because of the loss of the enzymatic activity. Subsequent measurements of the viscosity indicated that after 12 hours the values did not change. We observed the highest viscosity in the control sample, i.e., the sample without the enzymatic treatment, followed by the samples treated with 0.05% and 0.50% of polygalacturonase.

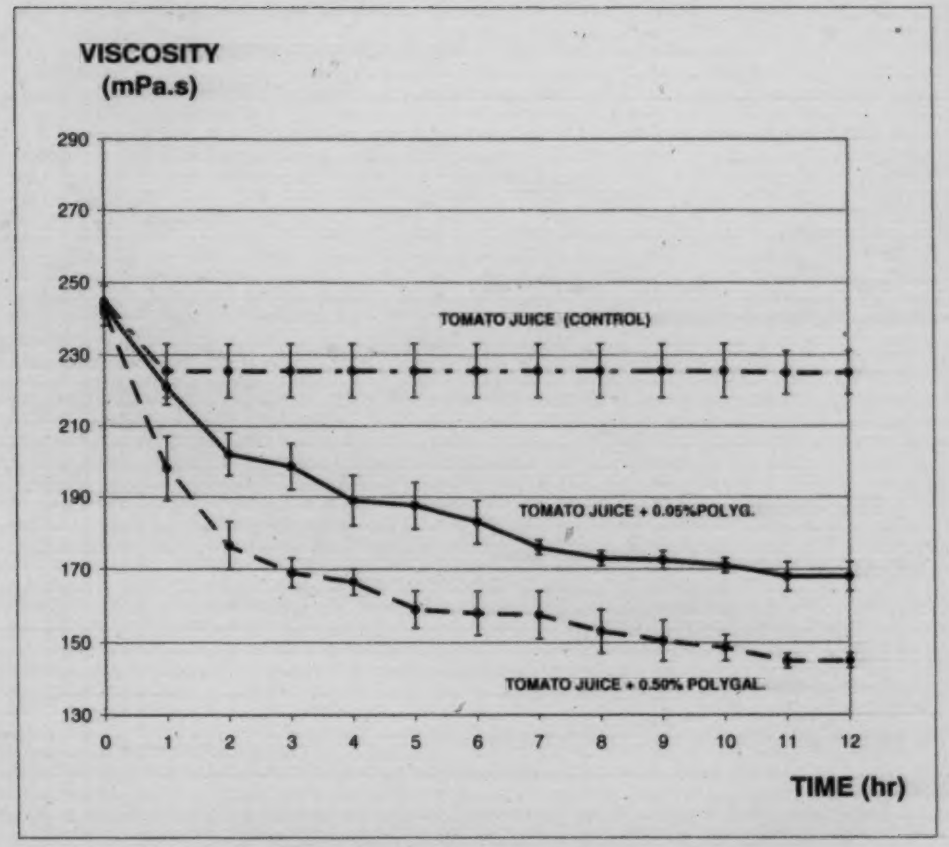

Figure 4.17: Viscosity of tomato juice at pH 4.2 treated with different percentages of polygalacturonase

In order to study the profile of viscosity changes of enzymatically treated tomato juice, samples of juice were prepared with additions of 0.05, 0.10, 0.15, 0.30, or 0.50% of polygalacturonase. The apparent viscosities of samples with enzyme as well as the control juice are depicted in Fig. 4.18. It can be noted that the highest viscosity corresponds to the control juice without enzyme followed by the samples with 0.05% up to 0.50% polygalacturonase. The viscosity among all samples showed to be significantly different ($p<0.05$). Subsequent analysis of the pectin content in samples confirmed the effect of the enzyme in the breakdown of the pectin as represented in the Table 4.2. The amount of pectin in the sediments of all samples was significantly different ($p<0.05$). Mutlu et al. (1999) reported a significant increase in the viscosity of a pectin solution with increasing pectin concentration. They also described the progressive drop in the pectin content of a pectin solution after the treatment with increasing concentrations of pectolytic enzymes ranging from 0.05% up to 2%. Our results are in agreement with their reports.

A mixture with 4% WPI and 96% tomato juice was selected as the system with a middle point concentration to determine the effect of the use of depectinized tomato juice in the blend. Mixtures containing 4%WPI and 96% tomato juice treated with polygalacturonase enzyme in 0.05, 0.10, 0.15, 0.30, or 0.50% concentration were prepared and the pH adjusted to 4.2. The effect, similar to that in pure tomato juice treated with the same enzyme, was a decrease in the viscosity with an increase in the amount of enzyme supplied, as depicted in Fig 4.18. The viscosity values for the control mixture (4%WPI and untreated tomato juice) and the samples with 4%WPI and enzymatically treated tomato juice are significantly different ($p<0.05$). The analysis of the pectin content of the 4%WPI and 96% tomato juice mixtures treated with polygalacturonase

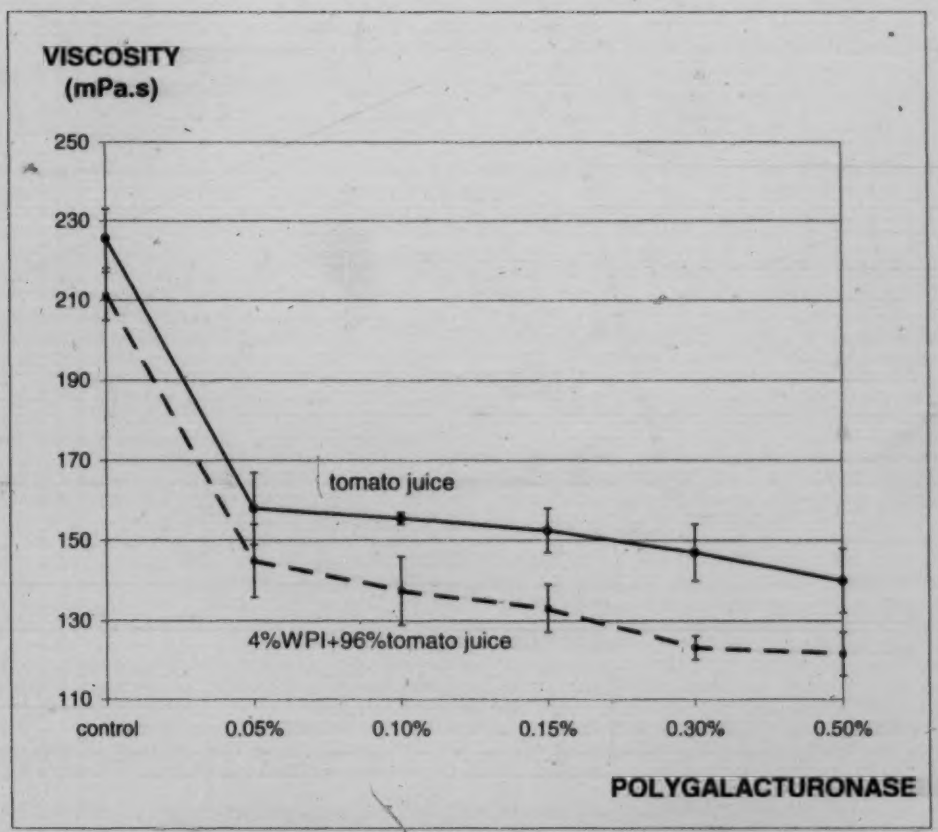

Figure 4.18: Viscosity of tomato juice and 4%WPI with 96% tomato juice mixture at pH 4.2 treated with polygalaturonase

Table 4.2 Relative pectin content in dry sediments of tomato juice at pH 4.2 treated with different amount of polygalacturonase (the data are expressed as mean ± standard error of the mean, n=4).

CONCENTRATION OF POLYGALACTURONASE	PECTIN CONTENT (%)
0%	9.55 ± 0.46
0.05%	8.37 ± 0.36
0.10%	6.73 ± 0.18
0.15%	6.27 ± 0.09
0.30%	5.64 ± 0.18
0.50%	5.09 ± 0.18

indicated that, as expected, the pectin content decreased noticeably with the increased concentration of enzyme added as showed in the Table 4.3.

Table 4.3 Relative pectin content in dry sediments of mixtures 4% WPI and 96% tomato juice at pH 4.2 treated with different amount of polygalacturonase (the data are expressed as mean ± standard error of the mean, n=4).

CONCENTRATION OF POLYGALACTURONASE	PECTIN CONTENT (%)
0%	2.07 ± 0.02
0.05%	1.49 ± 0.08
0.10%	1.49 ± 0.02
0.15%	1.29 ± 0.09
0.30%	0.94 ± 0.11
0.50%	0.94 ± 0.09

Although these results are in agreement with the tendency of the pectin content found in pure tomato juice (without WPI) treated enzimatically, the relative amount of pectin found in the WPI-tomato juice mixtures at each enzyme concentration is drastically lower ($p<0.05$). This could be because the enzymatic treatment of the juice left marginal amounts of "unbroken" pectin molecules to interact with the WPI protein

to form a complex. Thus, it may be assumed that the main component of the sediment must be protein in a high percentage.

4.3.4 Tomato juice-CCW mixtures

A mixture with 50% CCW and 50% tomato juice was produced in order to compare the prototype 4% WPI and 96% tomato juice with a mixture containing whey protein in a complex fresh whey system instead of purified whey protein concentrate. The proportion of CCW / tomato of this mixture was chosen based on the results of a previous sensory evaluation of a CCW-tomato juice drink carried out by Devkota (1991). In our experiments, two CCW-tomato mixtures at pH 3.3 and 4.2 were left for 18 hours at 20°C. Subsequent observation revealed that both mixtures presented many particles in suspension without any visual sedimentation.

After the solutions were heated at 90°C for 15 minutes and left to stand for another 18 hours, no changes in the red color were noted, and neither turbidity nor visual sedimentation of the particles in suspension appeared. The appearance was very similar to that of unheated mixtures. If some degree of sedimentation occurred it was extremely slow, probably owing to the small difference between the density of the liquid whey and the tomato juice. Additionally, the turbidity that may have developed was completely hidden by the deep color and natural suspended particles of the tomato juice.

Centrifugation of the unheated and heated mixtures at both pH levels was carried out to determine the amount of sediment. As depicted in Fig. 4.19, a slightly higher amount of dry sediment was obtained from the heated mixtures at pH 3.3, followed by pH 4.2. The sediments of unheated mixtures were also a little higher at pH 3.3 than at pH 4.2.

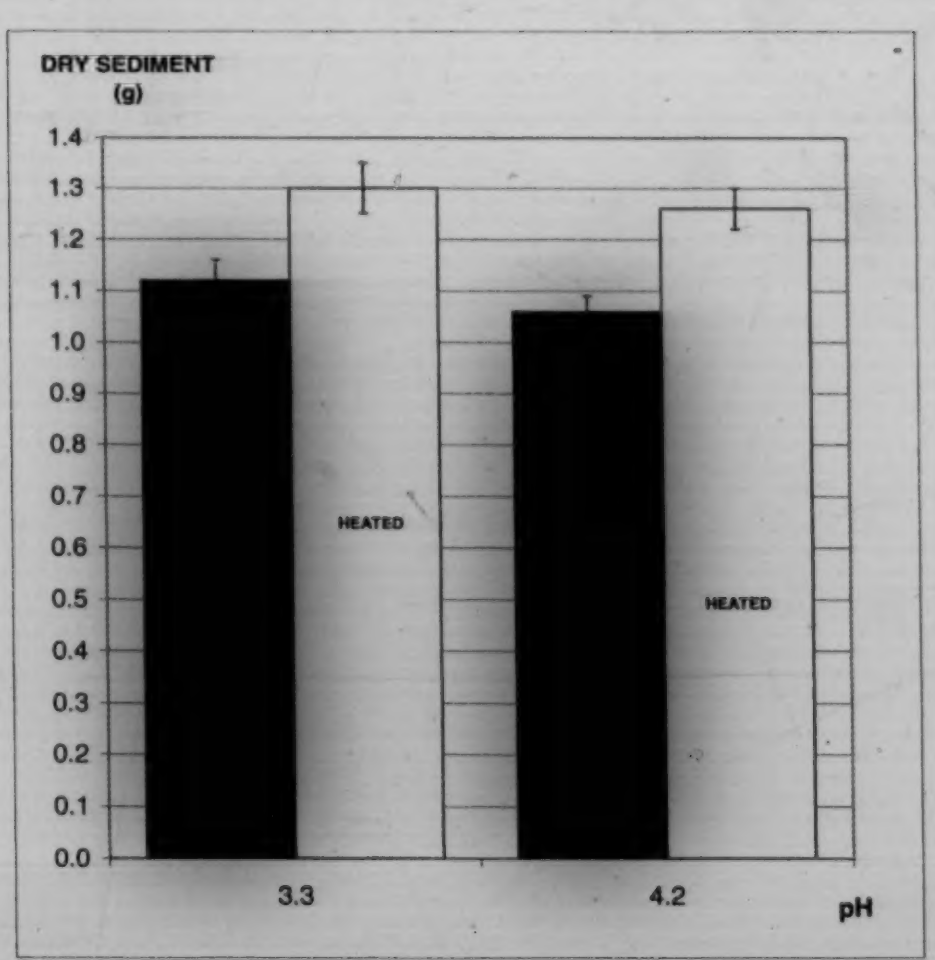

Figure 4.19: Dry sediments in 100 mL of unheated and heated mixtures of 50% CCW and 50% tomato juice

This showed a similar tendency with respect of the dry sediments obtained from unheated and heated mixtures of 6% WPI and 94% tomato juice (Fig 4.8 and 4.12). The amount of sediment from those mixtures was noticeably higher than the amount of sediment from unheated and heated CCW-tomato juice blends at both pH, due to the lower amount of whey protein in the CCW-tomato mixtures. The fact that the ionic strength of the tomato juice systems containing CCW is greater than those containing WPI, may count as an additional factor (Serov et all, 1985).

4.4 Drying of a tomato based prototype drink containing whey protein concentrate

According to our observations, the heating of solutions containing whey proteins in powder form mixed with tomato juice led to the coagulation of the whey proteins, and a noticeable increase in the viscosity and the amount of sediment obtained after centrifugation. Owing to these unfavourable effects, long-time or short-time preservation by the use of heat (pasteurization or sterilization) could be problematic. As an alternative, we decided to investigate spray drying the solutions to obtain a low moisture product, with high stability over time. Mixtures of 50% CCW and 50% tomato juice as well as 4%WPI and 96% tomato juice were prepared at pH 3.3 or 4.2 and spray dried.

While the spray drying of a mixture of 4% WPI-96% tomato juice was carried out easily, it was a different matter to spray dry the CCW-tomato juice mixture, as it produced a sticky wet powder. A similar adverse effect was obtained when the spray drying of a 100% tomato juice sample was attempted. Goula and Adamopoulos (2005a) reported that the spray drying of tomato pulp without the supply of extra dehumidified air in the intake of the dryer is difficult to carry out due to its

sticky nature. The powder stickiness is essentially caused by the low glass transition temperature (T_g) of the reducing low molecular sugars, mainly glucose and fructose, which constitute up to 65% of tomato solids (Miladi et al, 1969). On the other hand, Karatas and Esin (1994) stated that due to the reducing sugar presence in tomato paste and juice, a "case hardening" of the surface of the particles is developed during spray drying, a phenomenon resulting in the formation of an impermeable crust due to the progressive drying of the surface of the material when the rate of evaporation is higher than the moisture migration from the interior. The consequence is a discontinued falling rate period since hardening and plasticizing alternate in the surface of the droplets, causing a drying process difficult to control, with a variation in the quality of the final powder.

In fact, our mixture of 50% CCW and 50% tomato juice remained stuck on the wall of the drying chamber of the spray drier, with only very small amount of powder with a sticky and wet texture was recovered in the powder collector. An additional adverse factor, besides the reducing sugars of the tomato juice, is the presence of lactose in the CCW. Sienkiewicz and Riedel (1990) commented on the unfeasibility to spray dry acid whey if the lactose had not been crystallized. Consequently, a new mixture constituted by 48% CCW, 48% tomato juice, and 4% WPI at pH 3.3 and 4.2 was evaluated. This new mixture containing increased amount of total solids and higher ratio of whey protein-pectin was spray dried successfully, probably due to the effect of the added whey protein acting as a high molecular additive, which increased the T_g of the solution (Goula and Adamopoulos, 2005a).

4.4.1 Reconstitution of the powdered tomato based prototype drink

The total solids content of each mixture was determined before the spray drying process. Subsequently, the spray dried powders were reconstituted to solutions having these same amounts of original total solids: i.e., the same amount of water as was removed was added to the dried powder. The Table 4.4 summarizes the total solids of each mixture before drying, the moisture content of the spray dried powders, and the visual sediments of the reconstituted solutions. The reconstitution of each spray dried mixture was made by adding deionized water into the dry powder and stirring the solution at 20°C for 15 minutes.

The time for the dissolution of dry powders (or the time for the reconstitution) of 4% WPI-96% tomato and 48% CCW-48% tomato-4% WPI mixtures at pH 3.3 and 4.2, was measured by the addition of 2 g of powder into 50 mL of distilled water at 20°C under continuous stirring (El-Tinay and Ismail, 1985); the results are depicted in the Table 4.4. The dissolution time of powders obtained from the same mixture at pH 3.3 or 4.2 was not noticeably different, but the dissolution times of both types of mixtures were significantly ($p<0.05$) diverse. Goula and Adamopoulos (2005b) reported that powders with low moisture content appear to have fast dissolution, which is in accordance with our results. Our samples had lower times of dissolution (faster reconstitubility) than the values determined by Goula and Adamopoulos (2005b) for powders produced at the same air inlet temperature (130°C), although they were working with tomato pulp with 14% total solids. The presence of WPI in our mixtures and a different model of spray drier, in addition to different processing variables such as the feeding rate of product, the air inlet flow and the moisture of the inlet air, may be the cause of the differences in the solubility rates.

Table 4.4 Total solids, moisture of powder, visual sediments, and reconstitution time of WPI-tomato and CCW-tomato juice-WPI mixtures. (The data are expressed as mean ± standard error of the mean, n=4).

	4% WPI + 96% tomato juice		48% CCW + 48% tomato juice + 4% WPI	
% Total solids (before drying)	12.5 ± 0.13		10.8 ± 0.12	
pH	3.3	4.2	3.3	4.2
% Moisture of powder (after drying)	7.2 ± 0.04	7.3 ± 0.03	5.8 ± 0.16	5.4 ± 0.07
% Visual sediments (after reconstitution)	35.3 ± 3.15	16.0 ± 1.65	4.1 ± 0.90	69.5 ± 2.5
Reconstitution time (second)	92.5 ± 2.1	96.0 ± 1.5	78.0 ± 2.5	74.0 ± 2.0

After the reconstitution of the solutions, visual sediments were observed at pH 3.3 and 4.2 in both mixtures. The lowest amount of visual sediments was produced in the mixture CCW-tomato-WPI at pH 3.3 and the highest in the same mixture at pH 4.2. On the contrary, higher amount of sediments was observed at pH 3.3 than at pH 4.2 in the WPI-tomato juice mixtures. Since our previous observations demonstrated that the effect of the heating on the CCW-tomato and WPI-tomato mixtures

produced no visual sediment at both pH levels, the spray drying process may have produced some adverse effect in the whey proteins or pectin, perhaps decreasing the cohesivity of the network leading to sedimentation.

The final moisture content of the spray dried powders was slightly higher in the mixture 4% WPI-96% tomato than in the mixture 48% CCW-48% tomato-4% WPI. Since the operation parameters of the spray drier were kept the same for both mixtures, it may be possible that the higher amount of low molecular sugars in the mixture containing 96% tomato juice produced a drying process with lower efficiency, resulting in a powder with higher moisture content.

Considering the amount of variables involved in the spray drying of the whey-tomato juice mixtures in addition to the limitations of our laboratory-scale spray dryer, these results must be considered as a preliminary approach to demonstrate the feasibility of the spray drying of a WPI-tomato juice mixture. Determination of the best processing conditions is a further mandatory step to obtain a spray dry product with the highest quality.

5. CONCLUSIONS AND FUTURE PROSPECTS

5.1 Summary of Research Results

The overall goal of this research was the production of a tomato juice fortified with whey protein isolate. This necessitated a study of the interactions between high methoxy pectin and whey proteins in water models and real systems, the main focus of this work.

Solutions of whey protein in water ranging from 2% to 6% WPI showed no visual sedimentation at pH 3.3 and 3.7 even after heating, while at pH 4.2 visual sediments occurred in unheated and heated solutions. The visual sedimentation in heated solutions was significantly higher ($p<0.05$) than the sedimentation in unheated solutions. After centrifugation of the samples, a significantly higher ($p<0.05$) amount of dry sediment was obtained at all pH levels from heated solutions than from unheated ones.

The addition of 0.10%, 0.20%, or 0.30% pectin in 1% WPI solutions caused almost instantaneous visual sedimentation at pH 3.3 and 3.7, while no visual sedimentation occurred at pH 4.2. The amount of visible sediment was significantly greater ($p<0.05$) at pH 3.3 than at pH 3.7. Further centrifugation of the mixtures produced sediments at all pH levels, the highest amount at pH 3.3 and the lowest at pH 4.2. Following heating, the amount of centrifuged sediment increased considerably ($p<0.05$) at each pH, compared to unheated solutions. The highest amount of dry sediment at pH 4.2 was produced in the control solution having 1% WPI and no pectin.

Pectin analysis of the sediments after drying indicated that the amount of pectin increased with the increasing concentration of pectin in

the solutions at each pH. A slightly higher amount of pectin was found at pH 3.3 than at pH 3.7.

After heating, the pectin content in the centrifuged sediments was higher at pH 3.3 than at pH 3.7, following the same tendency as in unheated solutions. The pectin content in the sediments of heated blends was significantly lower ($p<0.05$) than the pectin content in the sediment of unheated solutions.

Mixtures of 2%, 4%, or 6% WPI and tomato juice at pH 3.3, 3.7 and 4.2, showed no visible sediment or turbidity at any pH or WPI concentration. After centrifugation, an increased amount of sediment was observed at all pH levels as the concentration of WPI increased. The highest amount of dry sediment was observed at pH 3.3 followed by pH 3.7 and pH 4.2 ($p<0.05$), a tendency previously observed in unheated WPI-pectin systems which demonstrates the protective effect of the pH in the sedimentation of the whey proteins-pectin complex. Subsequent heating produced considerably higher amount ($p<0.05$) of dry sediments than in unheated mixtures. The amount of dry sediment at each pH increased as the concentration of WPI rose from 2% to 6%. The highest volume of dry sediment after heating was observed at pH 3.3 and the lowest at pH 4.2.

Determinations of protein and pectin showed that with increasing concentration of WPI, the protein content of sediments increased, while the pectin content decreased. A significantly higher ($p<0.05$) protein content was found in sediments at pH 3.7 than at pH 3.3 and in an inverse trend, a considerably higher ($p<0.05$) pectin content was found at pH 3.3 than at pH 3.7. The protein content of sediment was drastically higher ($p<0.05$) in heated than in unheated mixtures, while the pectin

content was insignificantly ($p>0.05$) lower than the pectin content of unheated mixtures.

Single point measurement of the viscosity showed that at pH 3.3 and 3.7 the value of the viscosity rose as the WPI concentration increased, while at pH 4.2 the viscosity decreased with increasing amount of WPI in the mixture. Further measurements of the viscosity of mixtures with WPI concentration up to 15%, indicated that at pH 3.3 and 3.7 the viscosity rose continuously as the WPI concentration increased, while at pH 4.2, the viscosity dropped from 2% up to 8% WPI and increased from 10% to 15% WPI concentration. After heating, the highest viscosity was observed at pH 4.2 and the lowest at pH 3.3, and at each pH level the viscosity increased in direct proportion with the amount of WPI contained in the system.

Tomato juice samples treated with 0.05, 0.10, 0.15, 0.30, or 0.50% of polygalacturonase enzyme and a control sample without enzyme, showed higher pectin content in the sediments of the control sample, followed in consecutive order by the samples with 0.05% up to 0.50% of polygalacturonase. The pectin content of each sample was markedly different ($p<0.05$). Measurements of the viscosity showed the highest viscosity in the control sample, followed in decreasing order by the samples with 0.05% up to 0.50% polygalacturonase. The viscosity-time profile for 12 hours showed stable viscosity values in the control, while in the enzymatically treated samples, the viscosity decreased constantly up to 11 hours from the starting time.

The mix of 4% WPI and 96% tomato juice samples, previously treated with 0.05, 0.10, 0.15, 0.30, or 0.50% of polygalacturonase, showed reduction in the viscosity as the amount of enzyme increased. The viscosity of all 4% WPI-96% tomato juice mixtures treated with different concentrations of polygalacturonase, was significantly lower

($p<0.05$) than the viscosity of the pure tomato juice treated with the same amount of polygalacturonase. The analysis of the pectin content in the sediments of 4% WPI-96% tomato juice mixtures treated with polygalacturonase, indicated a noticeable drop ($p<0.05$) of the pectin content with the increased concentration of enzyme added. The amount of pectin found in the sediment of these mixtures at each concentration of enzyme added was noticeably lower ($p<0.05$) than the pectin content found in pure tomato juice treated with the same amount of polygalacturonase.

Mixtures containing 50% CCW and 50% tomato juice at pH 3.3 and 4.2 and left for 18 hours at 20°C, showed particles in suspension but no visual sedimentation. Heating produced no visible changes in the red color, and neither turbidity nor visual sedimentation of the particles in suspension. Centrifugation of the unheated and heated mixtures produced the highest amount of dry sediment in heated mixtures at pH 3.3. The amount of sediment of unheated or heated CCW-tomato juice mixtures was lower than the amount of sediment of unheated and heated 4% WPI-96% tomato juice mixtures at the same pH levels.

The spray drying of a 50% CCW-50% tomato juice mixture or a 100% tomato juice sample could not be carried out. Nevertheless, mixtures containing 48% CCW, 48% tomato juice and 4%WPI or 4% WPI and 96% tomato juice at pH 3.3 and 4.2 were spray dried successfully. Following spray drying, both mixtures showed good reconstitubility but visual sedimentation.

5.2 Conclusions

The previously described resistance of whey proteins to sediment at pH bellow 3.9 upon heating was confirmed by our investigation of the

sediment formation in aqueous solutions of 2%, 4%, or 6% WPI at different pH levels, as a baseline system. Without pectin, our whey protein product followed the established sedimentation resistance below pH 3.9.

This study confirmed that addition of pectin produced interactions leading to insoluble complex at pH 3.7 and 3.3 where pectin is reactive. At pH 4.2, pectin appeared to provide protection to the heat sensitive whey proteins. Our results confirmed that pectin and whey proteins react at pH level lower than 3.9, forming insoluble complexes that were visualized as sediment in WPI-pectin systems. Whey proteins will precipitate when heated below pH 3.9 and oppositely, will resist coagulation and sedimentation at pH 4.2 if pectin is present.

The WPI-pectin interactions were confirmed in the "real system" of WPI-tomato juice. The pectin reactivity in processed tomato juice and its complexation with whey proteins at the same pH levels above mentioned, was indirectly documented by measuring sediment formation, composition and solution viscosity. Undenatured whey proteins have the same effect when pectin is not reactive, at pH 4.2, on viscosity lowering in tomato juice as documented previously in a yoghurt drink (Patocka et al., 2006) because of the rearrangement of H_2O molecules in the complex system.

Following the heating of the WPI-tomato juice system, the viscosity increased noticeably. Since the viscosity of the tomato juice without WPI dropped after heating the juice, it can be proposed that the inverse effect in the WPI-tomato mixtures is caused because of the enhancement of the cohesivity of the network as a result of the increased aggregation between proteins and pectin.

The effect of pectin was confirmed by using polygalacturonase enzyme in the tomato juice which produced the breakdown of pectin

leading to less interaction with whey proteins, decreasing the viscosity in direct proportion to the amount of enzyme used in WPI and enzymatically treated tomato juice mixtures. The variation in the viscosity of the tomato juice treated with polygalacturonase, indicates the presence of pectin in the canned juice.

Addition of whey proteins had a beneficial effect in the spray drying of tomato juice, which otherwise is almost impossible to dry, and in combinations with CCW which is difficult to spray dry as well. Use of whey proteins can lead to new ways how to produce dried tomato juice, and contributes to further advances towards development of a whey drink containing nutraceutically superior tomato juice fortified with beneficial whey proteins. Visual sedimentation was noticeable following reconstitution of both spray dried solutions at pH 3.3 and 4.2, thus much further research is needed to make the dried tomato juice a success.

Taking into account the amount of variables involved in the spray drying, these results must be considered as a preliminary approach to demonstrate the feasibility of the spray drying of tomato juice products containing whey or whey components. The determination of the best processing parameters for each mixture and the selection of the suitable spray dryer, are mandatory prerequisites before a pilot scale production can be designed and further development of a WPI-tomato juice spray dried product conducted.

5.3 Recommendation for future research

Whey proteins and pectin have the ability to interact and produce complexes. The production of these complexes depends mainly on the concentration of proteins and pectin, the pH, the temperature and the ionic strength of the solution. Our experiments showed that the use of a

pectinase enzyme is able to modify the viscosity of a protein-pectin system, and the pectin content of the complex formed. A similar study could be carried out with regard to the degradation of the whey proteins and their effect in the formation of such complexes. Further investigation to elucidate the participation of each of the main whey proteins in the protein-pectin complex, by using isolated single proteins, would contribute to a better understanding of the protein-pectin interactions in a whey protein-tomato pectin system.

Single-point measurements of the viscosity of mixtures WPI-tomato juice at constant temperature indicated that the viscosity is influenced by changes in the pH, changes in the concentration of the WPI and tomato juice, and the time. However, the rotary viscometer used in our experiments was unable to give information about the flow behavior of these mixtures. Thus, following investigations to determine the rheological behavior of these systems, based in variable shear rate measurements, and the study of the way the network structure is affected by the pH, concentrations (WPI / tomato juice), and time, would be required.

The use of WPI or CCW mixed with tomato juice, produced tomato juice products with acceptable characteristics of color, appearance and sedimentation, and physical properties when the mixture was not heated. The application of heating as a necessary processing technique had a detrimental effect on the solubility, appearance and viscosity of the product. Therefore, more work is needed as heating (at least pasteurization) will likely be required in any industrial situation. Our study demonstrated the feasibility to spray dry a tomato juice fortified with whey proteins, although in a preliminary stage. An extensive study of the product preparation, pectin degradation treatment, spray drying process, and sensory evaluation of the reconstituted product has to be carried out

to lead to the development of a commercial tomato juice product containing a whey protein concentrate.

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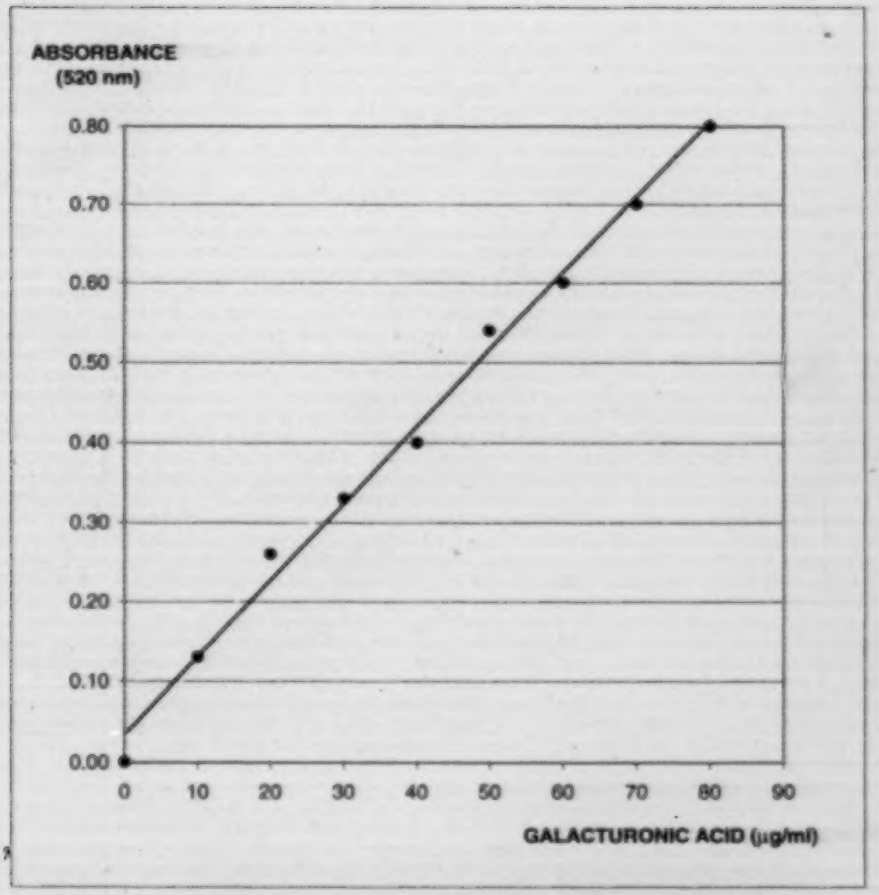

Appendix 1: Standard curve absorbance v/s galacturonic acid concentration (corrected by linear regression)